Title
The herpesvirus dUTPase encoded by KSHV's ORF54 facilitates the selective degradation of IFNAR1

Author
Farhat, Nisar Ali

Publication Date
2019

Peer reviewed|Thesis/dissertation

UNVERSITY OF CALIFORNIA

Los Angeles

The herpesvirus dUTPase encoded by KSHV's ORF54 facilitates the selective degradation of IFNAR1

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Molecular and Medical Pharmacology

by

Nisar Farhat

2019

ABSTRACT OF THE DISSERTATION

The herpesvirus dUTPase encoded by KSHV's ORF54 facilitates the selective degradation of IFNAR1

by

Nisar Farhat

Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2018

Professor Ting-Ting Wu, Chair

Through co-evolution herpesviruses have acquired selective strategies to exploit their hosts, effectively allowing life-long persistence in the host. Tumor-associated herpesviruses, also known as the gamma subfamily of herpesviruses, are distinct in their ability to establish latent infections in lymphocytes and cause benign or malignant tumors in the infected hosts. The two human gammaherpesviruses are Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). Given the critical role of type-I interferons (IFN α/β) in the host defense against viruses, it is not surprising that herpesviruses have encoded proteins to counteract the type-I IFN response. Our previous work employing the rodent gammaherpesvirus, murine gammaherpesvirus-68 (MHV-68) determined that a protein encoded by open reading frame (ORF) 54 counteracts the type-I IFN response, which aids the efficient establishment of lifelong persistent infection in the host (Leang, R.S., et al., 2011). In this dissertation, we elucidated the molecular mechanism by which the homolog protein encoded by KSHV's ORF54 antagonizes type-I IFNs and its biological significance in the context of infection. My thesis research led to two significant findings:

1. We identified a novel viral antagonistic mechanism of IFNs through targeting the endosomal trafficking of the interferon α/β receptor subunit 1 (IFNAR1).

2. We provide evidence to the hypothesis that this mechanism is evolutionarily conserved among all herpesviruses.

Explicitly we established lysosomal degradation mediated by the endosomal sorting complexes required for transport (ESCRT) machinery as the cellular pathway hijacked by ORF54 to down-regulate IFNAR1. Of note to this pathway is the necessary role of Ubiquitin Associated Protein 1 (UBAP1), a subunit of the ESCRT-I complex, for ORF54 to shuttle endocytosed IFNAR1 towards lysosomes. Moreover, we showed that ORF54 does not induce cellular signals that are known to promote IFNAR1. Indeed, ORF54 does not affect IFNAR1 internalization. This feature distinguishes ORF54 from other known viral mechanisms that induce IFNAR1 degradation.

Our data indicate that ORF54 interacts with UBAP1 as well as with IFNAR1, serving as an adaptor to recruit the endocytosed receptor protein to the ESCRT machinery. Ubiquitination of endocytosed proteins is a critical sorting signal for ESCRT. Because ORF54 can bridge the interaction between IFNAR1 and UBAP1 without IFNAR1 being ubiquitinated, it facilitates the introduction of any endocytosed IFNAR1, ubiquitinated or not, into the ESCRT-mediated lysosomal degradation pathway. Furthermore, we showed that another cell surface protein, epidermal growth factor receptor (EGFR), while also undergoing constitutive endocytosis as IFNAR1, is not targeted by ORF54. And yet, IFNAR1 is not the only cell surface receptor targeted by ORF54; we found that gp130 can be down-regulated as well. Therefore, we conclude that ORF54 can selectively target endocytosed surface proteins and bypass vital regulatory steps that mark proteins for degradation.

ORF54 is a functional 2'-deoxyuridine 5'-triphosphate pyrophosphatase (dUTPase) and we previously showed that this enzymatic activity is not required for ORF54 to down-regulate IFNAR1. All herpesviruses, alpha-, beta-, and gamma subfamilies, encode orthologs dUTPases. However, the dUTPase of betaherpesvirus does not have nucleotide hydrolyzing activity. We demonstrated that dUTPases of all

three subfamilies interact with UBAP1 and down-regulate IFNAR1. My thesis work has uncovered an evolutionarily conserved viral mechanism antagonizing the innate immune response, suggesting a novel, critical role of herpesviral dUTPases for the establishment of persistent infection and viral pathogenesis.

The dissertation of Nisar Farhat is approved.

Caius Gabriel Radu

Steven J. Bensinger

Gregory S. Payne

Ren Sun

Ting-Ting Wu, Chair

University of California, Los Angeles

2019

DEDICATION

For my sons, I may not have had the privilege of being your father in this lifetime, but I hope to have the opportunity to teach you all I know in the next.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ACKNOWLEDGEMENTS

The work for this dissertation was performed under the direction of Dr. Ting-Ting Wu. I would like to express my appreciation for her guidance and patience. I would also like to thank my committee for the continued support they have granted me.

VITA

Education

2011	B.S., Chemistry and Biochemistry
	California State University, San Bernardino (CSUSB)
	San Bernardino, CA, USA
2013	M.S., Bioengineering
	University of California, Riverside (UCR)
	Riverside, CA, USA

Publications and presentations

Farhat, N.*, Brar, G*, Sukhina, A., Lin, W., Kim, Y.H., Hsu, T., Ware, C. Blackman, M.A., Sun, R., Wu, T.T. UBAP1 is critical for selective lysosomal degradation of IFNAR1 by KSHV's ORF54. (*Manuscript in preparation)* (*authors contributed equally).

Farhat, N.*, Brar, G*, Sukhina, A., Lin, W., Kim, Y.H., Hsu, T., Ware, C. Blackman, M.A., Sun, R., Wu, T.T. UBAP1 is critical for selective lysosomal degradation of IFNAR1 by KSHV's ORF54. (*Manuscript in preparation)* (*authors contributed equally).

Gong, D., Kim, Y.H., Xiao, Y., Du, Y., Xie, Y., Lee, K.K., Feng, J., **Farhat, N.**, Zhao, D., Shu, S., Dai, X., Chanda, C.K., Rana, T.M., Krogan, N.J., Sun, R., Wu, T.T. A Herpesvirus Protein Selectively Inhibits Cellular mRNA Nuclear Export. *Cell Host & Microbe* **20,** 642–653 (2016).

Annual Molecular and Medical Pharmacology Retreat 2018: Rerouting membrane proteins: A novel mechanism of interferon antagonism. November 2018. Oral Presentation

Annual Molecular and Medical Pharmacology Retreat 2017: KSHV's ORF54 selectively targets IFNAR1 towards lysosomal degradation. November 2017. Oral Presentation

Annual Molecular and Medical Pharmacology Retreat 2016: KSHV's ORF54 selectively targets IFNAR1 towards lysosomal degradation. November 2016. Poster Presentation

19th International Workshop on KSHV and Related Agents 2016: Gammaherpesvirus Open Reading Frame 54 selectively shuttles IFNAR1 towards lysosomal degradation. July 2016. Oral Presentation

International Herpes Workshop 2016: Gammaherpesvirus Open Reading Frame 54 selectively shuttles IFNAR1 towards lysosomal degradation. July 2016. Oral and Poster Presentation

Annual Molecular and Medical Pharmacology Retreat 2015: Open Reading Frame 54 selectively shuttles IFNAR1 towards lysosomal degradation. November 2015. Poster Presentation

International Herpes Workshop 2015: Mechanism of Interferon Dysregulation by ORF54. July 2015. Oral Presentation

Annual Molecular and Medical Pharmacology Retreat 2014: Mechanism of Interferon Dysregulation by ORF54. November 2014. Poster Presentation

Introduction

Kaposi's Sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) are the two members of human gammaherpesvirus family[1-5]. Like all herpesviruses, these are large, double stranded DNA viruses that undergo a bi-phasic lifecycle resulting in the persistent infection of the host, called latency[1-5]. As members of the gamma subfamily, these viruses establish latency in B-lymphocytes, and largely coexist with their human host without causing disease[6]. Under normal conditions, the immune system carefully monitors and limits the proliferation of these KSHV and EBV by recognizing viral proteins (antigens)[6]. If this process fails due to an abnormal immune response, the chances of virally induced cell proliferation increase, resulting in malignancies associated with KSHV and EBV[6]. To efficiently maintain a persistent infection of the host, these viruses dedicate a large portion of the viral genome to the evasion of the immune system[7]. During lytic replication, specific immune modulatory proteins subvert and exploit the antiviral activities of the cell intrinsic immune response, as well as those of the innate and adaptive immune cells[7-8]. During latent infection, the virus cleverly restricts the expression of genes without production of virions and reduces the presentation of latency-associated proteins to major histocompatibility complex (MHC), thereby avoiding immune elimination[7-8]. This chapter serves as an overview of human gammaherpesvirus biology and immune evasion.

Background

Virion and Entry. The Herpesvididae family is a group of large double-stranded DNA viruses with broad species tropism[1-6]. Impacting human beings are eight members of this family, divided into three subgroups; namely, alpha-, beta-, and gammaherpesviruses (listed in Table 1). Constituting the alpha-herpesviruses subgroup are herpes simplex 1 (HHV1) and 2 (HHV2) the

causative agent of cold sores and genital herpes, as well as the Varicella Zoster virus (VZV or HHV-3), responsible for chickenpox. Members of the betaherpesvirus subgroup include cytomegalovirus (CMV) and human herpesvirus 6 and 7[7]. The human gammaherpesvirus family include two members, Kaposi's Sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV). Unlike the alpha and beta subgroups, gammaherpesvirus are associated with various malignancies[6]. Of note is that unlike EBV, which is ubiquitously observed among human population, KSHV's seroprevalence varies among geographical locations. The work of this dissertation focuses on KSHV.

Like all herpesviruses, the KSHV virion is surrounded by a lipid bilayer. This viral envelope is studded with virally-encoded glycoproteins gB, gG, gM, gL, and gN, ORF68, and K8.1[13-18]. Immediately following the viral envelope is the protein filled space that exists between the envelope and the capsid. The tegument contains viral proteins including ORF21, 33, 34, 63, 64, and 75, as well as 11 viral RNA transcripts. KSHV's capsid is an icosahedral structure composed of repeating patterns of five viral ORFs. These include the major capsid protein ORF25, ORF62, 26, and 17.5, and the small capsid protein ORF65[3,5]. KSHV's genome consists of linear double-stranded DNA which circularizes upon entry of cells. Approximately 140 kb of the viral genome are unique coding sequences flanked by 25-30 kb of repetitive terminal repeats. KSHV's ORFs are numbered from the left end genome beginning with ORF1 and running to the right ending in ORF75. ORFs unique to KSHV carry a "K" in the designation, such as K1. Alongside protein-coding genes, the genome also encodes for microRNAs and other non-coding RNAs[3,5].

Two phases of the viral life cycle. Upon entry, proteins in the tegument contribute to changing the cell signaling pathways to alter the cytoskeleton and deliver the capsid to the nuclear periphery where it uncoats and deposits the viral genome to the nucleus[3]. All herpesvirues have

two distinct phases of the life cycle: latency and lytic replication. Upon infection of permissive cells, KSHV mostly enter the latency phase where only a limited repertoire of viral genes are expressed primarily originating from the latency locus of the genome. In contrast, lytic replication infrequently occurs after infection[3]. Reactivation of latent KSHV into lytic replication can be induced by a variety of signals, including cytokine signaling, cell differentiation, reactive oxygen species, and innate immune signaling by toll-like receptors (TLRs)[7]. The viral transactivator, RTA, initiates a complex transcriptional cascade that results in the expression of all viral genes, replication of the viral genome, and the subsequent assembly, egress, and release of progeny virions[1-3]. Although spontaneous lytic replication is observed at different levels among KSHV-associated malignancies, the majority of infected cells remain latently infected. This observation suggests a primary role of the latent viral proteins in KSHV tumorigenesis[6].

During latent infection, the circularized viral genome persists as episomes without integrating into cellular chromosome. The latency-associated nuclear antigen (LANA) tethers the viral episome to the host's chromosome by concurrently binding terminal repeats on the viral genome and host histones H2A and H2B[19]. Via the host's DNA replication machinery the viral genome is amplified and passed along to both daughter cells with each cell division - thus persisting. The latency locus encodes LANA, vFLIP, vCyclin, kaposin, and microRNAs[20-21]. The LANA promoter controls the expression of LANA, vCyclin, and vFLIP, while the kaposin promoter drives the expression of three kaposin transcripts, a bicistronic transcript for vCyclin and vFLIP, and the twelve viral pre-miRNAs[20-21]. Of note are the phenotypes of transgenic mice that express some or all of the KSHV latency locus exhibiting characteristics of KSHV malignancies, further emphasizing the transformative potential of KSHV latency genes[6].

KSHV Transmission. In endemic areas where seroprevalence is high and seroconversion occurs at young ages, KSHV appears to be primarily transmitted via saliva[22]. In

areas where KSHV prevalence is less than 1%, sexual contacts are the major route of transmission. Transmission by blood or blood products, or transplantation of solid organs also occurs[6,22]. In addition to B-cells, KSHV has been detected in endothelial cells, epithelial cells, and monocytes. In culture, however, the virus can infect a wider variety of cells variety of cells including fibroblasts, keratinocytes, B lymphocytes, monocytes, plasmacytoid dendritic cells (pDCs), endothelial cells, and epithelial cells, suggesting that KSHV may be found in supplementary tissues but remains below the level of detection[6-7].

KSHV's glycoproteins facilitate fusion of the virus to permissive target cells. Of note, gB, gH, and gpK8.1A bind heparin sulfate likely facilitating the association to cellular receptors and viral entry. Additionally, gB contains an RGB integrin-binding motif[14-17]. This observation is of particular interest since αVβ3, and αVβ5 integrins have been shown to play a role in viral entry[14]. Other cell receptors that bind to KSHV include the 12- transmembrane glutamate/cysteine exchange transporter protein xCT[18], as well as the cell-specific intercellular molecule 3 (ICAM)-grabbing non-integrin (DC-SIGN) found on B-cells, DCs, and macrophages[13]. Once bound, KSHV primarily enters cells via clathrin-mediated endocytosis or macropinocytosis, and is translocated in the cell via the endosomal sorting complex required for transport (ESCRT)[13,23].

KSHV Innate Immune Evasion

The human immune system function in recognizing and eliminating invading pathogens. Upon infection of the host, viruses are faced with overcoming the host's immune response. Constituting the first layer of defense, the innate immune response is comprised of layers of non-specific defenses, including anatomical barriers, such as skin and mucosa, the complement system, inflammation, and various cell types, such as natural killer cells, phagocytes, mast cells, macrophages, dendritic cells, neutrophils, and basophils[7-12].

The innate immune response is dedicated to the detection as well as the elimination of pathogens and is critical in shaping and the ensuring, long-term, adaptive immune response. This task is completed in part via the signaling of cytokines, such as type-I interferons (IFNα/β). These cytokines are secreted upon viral infection and induce the expression of a variety of antiviral gene products, aimed at reducing virus replication and limiting the spread of infection[24-27].

Priming by the innate immune response leads to the activation of the adaptive, or specific, immune response. This second-tier action by the host's immune system is characterized by the production of antibody responses and cell-mediated responses, which are carried out by different lymphocyte cells, B cells and T cells, respectively[24-27]. Antibodies, which circulate in blood plasma and lymph, bind specifically to foreign antigens and are principally produced by B cells. Cell-mediated immunity involves the activation of macrophages, natural killer cells (NK), antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to an antigen. Vital to the host's long-term immunity against foreign pathogens is the development of immunological memory, in which pathogens are “remembered” by a signature antibody[24-27].

While KSHV is the causative agent of tumorigenesis, the state of the host's immune system is pivotal to the fate of the clinical outcome of KSHV-associated diseases[6-12]. This phenomenon is demonstrated by the increased replication and enhanced malignant progression in immunocompromised individuals, such as AIDS patient or transplant recipients[6-12,22]. Viral persistence in healthy, immunocompetent hosts suggests a critical equilibrium between the KSHV and the immune system[6-12]. Thus, essential to the viral persistence is the ability to evade host immune recognition and attack that would result in virus elimination. To overcome these

challenges, KSHV has evolved a sophisticated repertoire of viral genes and effective mechanisms dedicated to overwhelming or co-opting the host's immune response[6-12].

Immune Activation. Viral infection of the host leads to the production of type-I Interferons (IFNs) and pro-inflammatory cytokines[24-27]. This essential induction of innate immune signaling plays a vital role in the immune surveillance by sensing pathogens and initiation protective immune responses. Initiating inflammatory responses are receptors that recognize pathogen-associated molecular patterns (PAMPs) and induce NFκB signaling and production of type I IFN and proinflammatory cytokines[24-27]. The responsible receptors/sensors belong to one of five types of pattern-recognition receptors (PPRs): Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and the AIM2-like receptors (ALRs)[24-27]. To evade the host's immune response, KSHV targets key regulatory steps of the host innate immune response, including the IFN-mediated antiviral immunity[6-12,24-27].

Evidence has shown that TLRs 3, 4, 7, 8, and 9 are involved in viral recognition by binding to RNA, DNA, or viral glycoproteins[26-28]. Thus, there exists an initial TLR-mediated innate immune response to KSHV primary infection, which is subsequently abrogated by the virus[6-12]. Mounting evidence implicates KSHV modulation of TLR signaling by influencing the expression of TLRs or their adaptor molecules. Infection of monocytes leads to an upregulation of TLR3 and downstream innate immune effectors[29]. In contrast, infection of endothelial cells results in downregulation of TLR4 mediated by the lytic proteins vIRF1 and ORF74[30]. Furthermore, RTA a viral E3 ubiquitin ligase efficiently antagonizes TLR2 and TLR4 signaling in THP-1 monocytes by causing their degradation[31-33]. RTA additionally induces the degradation of TRIF indirectly, thereby preventing TLR-3 signaling[31-33]. RTA is also responsible for the

proteasomal degradation of MyD88, thereby repressing TLR4 signaling and mediated innate immunity[34]. This evidence facilitates RTA's role as a critical virulence factor that antagonizes the TLR-mediated type-I IFN response. KSHV microRNAs (miRNAs) miR-K9 and miR-K5 regulate TLR/IL-1R signaling cascade by mediating the down-regulation of IRAK1 and MyD88[35].

Studies indicate that KSHV targets other PRRs in addition to TLRs. Inn et al. show that the tegument protein, ORF64, targets explicitly and suppresses RIG-I-mediated signaling via its deubiquitinase activity[36]. Furthermore, West et al. recently showed that in cells depleted of either MAVS or RIG-I, KSHV infection increases global transcription of the viral genome during de novo infection[37]. This indicates that both MAVS and RIG-I inhibit KSHV transcription after primary infection. Gregory et al. report that another tegument protein, ORF63, is a functional homolog of NLRP1 that disrupts the formation and activity of the NLRP1 inflammasome[38]. While NLRP1 is a cytoplasmic inflammasome, a study by Keruer et al. shows that, during KSHV infection of endothelial cells, IFN-γ-inducible protein 16 (IFI16) interacts with a caspase-1 activating complex to form a functional nuclear inflammasome, suggesting that KSHV may manipulate this pathway to promote latency after nuclear delivery of the viral genome[39]. These recent studies indicate a broader role for PRRs in sensing γ-herpesvirus infection and suggest that additional viral antagonists and strategies remain to be identified.

The second wave of the innate immune response that results in the expression of the type-I interferons (alpha/beta). The release of these pro-inflammatory cytokines leads to an autocrine and paracrine response resulting in the expression of interferon-stimulated genes (ISGS), as well as genes that suppress cell growth, promote apoptosis, enhance antigen presentation, and modulate essential signal transduction pathways. Upon binding its ligand, the type-I interferon

receptor (IFNAR) subunits 1 and 2 dimerize leading to the initiation of a signaling cascade that results in the expression of ISGs and the mounting of an antiviral response. KSHV antagonizes both the expression and the function of different components of the type-I IFN response[24-28].

KSHV encodes four homologs of cellular IRFs, named viral IRF1-4 (vIRF), which actively interfere with the transactivation activity of specific host IRFs[40-41]. vIRF1 inhibits the formation of the transcriptionally active IRF3-CBP/300 complex[42-44], while vIRF2 associates with protein kinase R (PKR), limiting its antiviral activity[45-47]. vIRF3 antagonizes the master regulator of type-I IFN production IRF7, as well as IRF5 via direct inhibitory interaction[48-49], while vIRF4 appears to enhance the degradation of p53 may not be interfering with IFN-mediated signaling. vIRFs also inhibit TLR3-dependent IFN signaling[50]. Although these proteins display homologous functional activities, they appear to be differentially expressed, suggesting distinct functions at different time points of infection or in different cell-type[51].

KSHV's LANA (ORF73), and K-bZIP (K8), competitively bind to the IFN-β promoter, preventing the induction of INF-beta[52]. The viral ubiquitin ligases K3 and K5, also known as modulators of immune recognition (MIR)1 and MIR2, are highly homologous and encode a RING-CH domain[53-54]. These viral proteins function to promote the ubiquitination, endocytosis, and degradation of the IFN-gammaR1 subunit of the IFN-gamma receptor via lysosomal degradation. Mutational analysis indicates that K5 appears to down-regulate IFN-gammaR1 more strongly than K3[55]. K5 is also involved in the proteasomal degradation of tetherin[56]. Furthermore, K3 and K5 also mediate the proteasomal degradation of MHC-I, thereby preventing the antigen presentation[57-59]. While MHC-I degradation appears to be preferentially accommodated by K3, K5 also facilitates the down-regulation of ICAM (CD54) and B7-2

(CD86), co-activating proteins present on B-cells that stimulate activation of NK cells[60-62]. Taken together, it appears that K5 functions to prevent detection by CTL and NK cells[60-62].

KSHV's extensive repertoire of viral proteins that coordinate to manipulate and antagonize host innate and adaptive immunity, lead to the successful establishment of viral latency. Due to the lack of permissive cell cultures and strong *in vivo* model systems for KSHV, there remains essential research that needs to integrate observations regarding KSHV innate immune antagonists and the sophisticated molecular mechanisms employed by these viral immunomodulatory proteins.

Regulating IFNAR1 – An overview.

The type-I interferon response presents the first immunological barrier to pathogens have to overcome to affect a productive infection of the host. In more detail, upon binding its ligand the IFNAR1 and IFNAR2 subunit of the type-IFN receptor (IFNAR) bind and dimerize6[6,63]. To date, 14 isoforms of interferon alpha and a single isoform of interferon beta have been identified. Although both cytokines initiate a signaling cascade, differences in their binding and signaling capabilities exist[64-65]. The members of interferon alpha isoforms require the binding of to IFNAR2 first before facilitating the dimerization of the receptor by binding IFNAR1. Due to a smaller surface area binding site the association between interferon alpha and IFNAR1 is unstable. In contrast, interferon beta can stably bind IFNAR1 and induce the homodimerization of the receptor. This association allows the cytokine an additional avenue for signaling through the IFNAR receptor[64-65].

Receptor engagement activates IFNAR1-associated Tyrosine kinase 2 (Tyk2) and IFNAR2-associated Janus kinase 1 (Jak1)[66]. These kinases subsequently regulate the phosphorylation and activation of different STAT proteins. Activated STAT proteins homo- or

heterodimerize and translocate to the nucleus, where they promote the expression of numerous target genes[66-67]. In addition, type I IFNs activate the MAPK, PI 3-K-Akt, and NF-kappa B signaling pathways[63]. One transcriptional complex that is formed following stimulation by type I IFNs is the IFN-stimulated gene factor 3 (ISGF3) complex. This complex consists of phosphorylated STAT1, STAT2, and IRF9 and binds to IFN-stimulated response elements (ISREs) found in the promoters of numerous IFN-stimulated genes (ISGs). Another STAT homo- or heterodimers induced by type-I IFNs bind to regulatory sequences in the promoters of target genes known as IFN-gamma-activated sequence (GAS) sites. Binding of STAT proteins to either ISREs or GAS sites regulates the expression of several hundred ISGs, which mediate the anti-viral, antiproliferative, and apoptotic effects of type I IFNs[63].

Via regulation of the IFNAR1's expression on the plasma membrane, the cells regulate the strength and transience of the pro-inflammatory signal[63,68]. The half-life and surface level of a plasma membrane protein, such as a cytokine receptor, is determined by the rates of internalization, recycling, and degradation[63,68]. Therefore, cellular responses to extracellular environment stimuli can be regulated by endocytic trafficking of the responding receptor to modulate its surface expression. Following ligand binding accelerates IFNAR1 internalization and its sorting to degradation, leading to down-regulation of surface expression of the receptor. Upon IFN binding, IFNAR1 is phosphorylated on serines 535 and 539 by its associated kinases[68-69]. Consequently, biochemical and structural changes expose the degradation domain on the cytoplasmic tail of IFNAR1 which acts as the docking site to recruit SCFHOS Ring E3 ligase complex, which subsequently ubiquitinates IFNAR1[70-71]. Ubiquitination of IFNAR1 increases its internalization and endocytic sorting towards lysosomal degradation. This ligand- and ubiquitin-dependent endocytosis and degradation represents a significant mechanism to terminate the signaling from cell surface receptors.

Like many other membrane receptors, IFNAR1 also undergoes ligand-and ubiquitination independent basal level internalization[72]. This constitutive recycling of the receptor facilitates tight regulation of the type-I interferon induction strength at a minimal metabolic cost to the cell[72]. IFNAR1-associated Tyk2 plays an essential, albeit paradoxical, role in maintaining the surface expression of IFNAR1 by preventing the association of the AP-2 complex[73-74]. Limiting the access of AP-2 prevents clathrin-coated endocytosis of IFNAR1[73-74]. It is generally believed that recycling of endocytosed cell surface proteins is mediated by bulk membrane flow. However, it remains to be determined whether recycling of IFNAR1 is regulated by a specific sequence-dependent pathway[68,72-74].

Regulation of the type-I interferon response through IFNAR2 is less well characterized. Research indicates that the cell produces three isoforms of the IFNAR2 subunit. First identified, isoform a is functionally relevant with a complete cytoplasmic tail that associates with Jak1. Isoform b appears to be missing a cytoplasmic tail and thus binds ligand but is unable to propagate the necessary signal[67,75-77]. Therefore, the function of isoform b is hypothesized to sequester ligand, thereby limiting the propagation of the pro-inflammatory signal. Isoform c is serum soluble, and its function remains elusive[76]. It has been proposed that its role in circulating in serum is to bind and sequester cytokines, this has not been experimentally linked to an immune regulatory phenotype[76]. The transcriptional regulation exerted by the IFNAR2 isoform expression suggests that it functions later in the abrogation and regulation of the type-I IFN response. Thus, IFNAR1's sequestration represents an immediate shut-off to the prolonged type-I IFN signal, and IFNAR2 isoform expression further reinforces the negative feedback. In concert, the tight regulation of the IFNAR receptor is essential to an appropriate pro-inflammatory signal that leads to the elimination of pathogens.

Viral dUTPase – The discovery of novel interferon antagonists

The type-I IFN response represents an immediate, essential barrier to viral infection of the host. Thus, overcoming this barrier is a critical step in the viral life cycle and successful infection resulting in infectious progeny virions. A virus that is deficient in evading type-I IFN responses should be highly attenuated in a host. As part of a larger study that strategically designed a live attenuated vaccine candidate, our lab performed an unbiased viral ORF library screen to systematically identify viral antagonists of type-I IFNs. Due to the restrictive host specificity of KSHV, our lab has been utilizing the rodent homolog virus of KSHV, murine gammaherpesvirus-68 (MHV-68), as a model for the in vivo studies of gammaherpesvirus pathogenesis and immune modulation. The screen was performed on MHV-68 and established a foundation for the successful development of a proof-of-concept vaccine strategy as part of the thesis work by Dr. Gurpreet Brar.

The screen results revealed a set of four structurally related proteins that function to antagonize the IFN-I response. This set of genes, ORF10, ORF11, and ORF54, share a dUTPase-related domain. Our previous work by Leang et al. established ORF54 as an enzymatically active dUTPase. dUTPases are involved in hydrolyzing dUTP to prevent the mis-incorporation of uracil into the DNA. Leang et al.[78] characterized the impact of MHV-68 ORF54 on the suppression of the type-I IFN response. The work identified that both MHV-68 and KSHV's ORF54 reduces the IFN-induced activation of a reporter based on the promoter containing interferon stimulatory response element (ISRE). The anti-IFN function of MHV-68 ORF54 is independent of its dUTPase activity and is not found in the cellular dUTPase. This phenotype makes ORF54 a multi-tasking protein, as is often the case for viral proteins that possess multiple functions due to the limited genomic space. ORF54 expression leads to the degradation of

IFNAR1 that results in the reduction of the type-IFN response as measured by the induction of phosphorylated STAT1 (pSTAT1). In the context of MHV-68 infection, the IFN-induced pSTAT1 is reduced and partially restored in the infection of a mutant virus lacking the expression of ORF54 via a stop codon mutation (54S), despite that multiple viral anti-IFN proteins were found. In addition, IFNAR1 is down-regulated in cells infected with the wild-type (WT) virus and this phenotype is mostly abolished in cells infected with 54S. Infection of bone marrow derived macrophages (BMDMs) with 54S yields a higher induction of ISGs compared to WT. Accordingly, viral gene expression of 54S in BMDMs is reduced and rescued in cells lacking IFNAR. The replication of 54S in mouse fibroblasts is more sensitive to IFN treatment than WT. Infection of mice with 54S leads to a significantly lower viral latent load compared to WT. Critically this phenotype is rescued in IFNAR-deficient mice, linking the evasion of type-I IFN responses to the establishment of latency. This is significant since latency is directly linked to tumorigenesis associated with human gammaherpesviruses.

It remains unclear how ORF54 mediates IFNAR1 down-regulation. In addition, work by Madrid et al.[79] has found other surface molecules down-regulated by KSHV's ORF54 in addition to IFNAR1, such as gp130, IL-23R, IFNGR1, and NKp44L while EGFR remains unperturbed. Of note is the difference in research employed by these two studies. While Leang et al. evaluated the loss of total IFNAR1 via western blotting, Madrid et al. only reported the reduction of surface expression. Furthermore, Leang et al. demonstrated the impact of ORF54 on the induction of type-I IFN response and its functional significance in both in vitro and in vivo studies. Madrid et al. instead focuses on the evaluation of NKp44L, which remains of unknown identify, and its functional inhibition by assessing activation of natural killer cells. However, they did not evaluate the total cell surface protein level, thus concluding that ORF54 induces the sequestration of selective innate immune membrane proteins and cytokine receptors in the

cytoplasm. Moreover, they failed to demonstrate the role of ORF54 in the reduction of NKp44L during KSHV infection. Therefore, the focus of this dissertation is to further understand the discrepancies between these two studies and elucidate the molecular mechanism involved in the IFNAR1 down-regulation by KSHV's ORF54 in details. As the surface level of IFNAR1 is critical for signaling and tightly regulated, we believe that our study will also generate novel insights into the cellular pathways that control IFNAR1 expression on the cell surface.

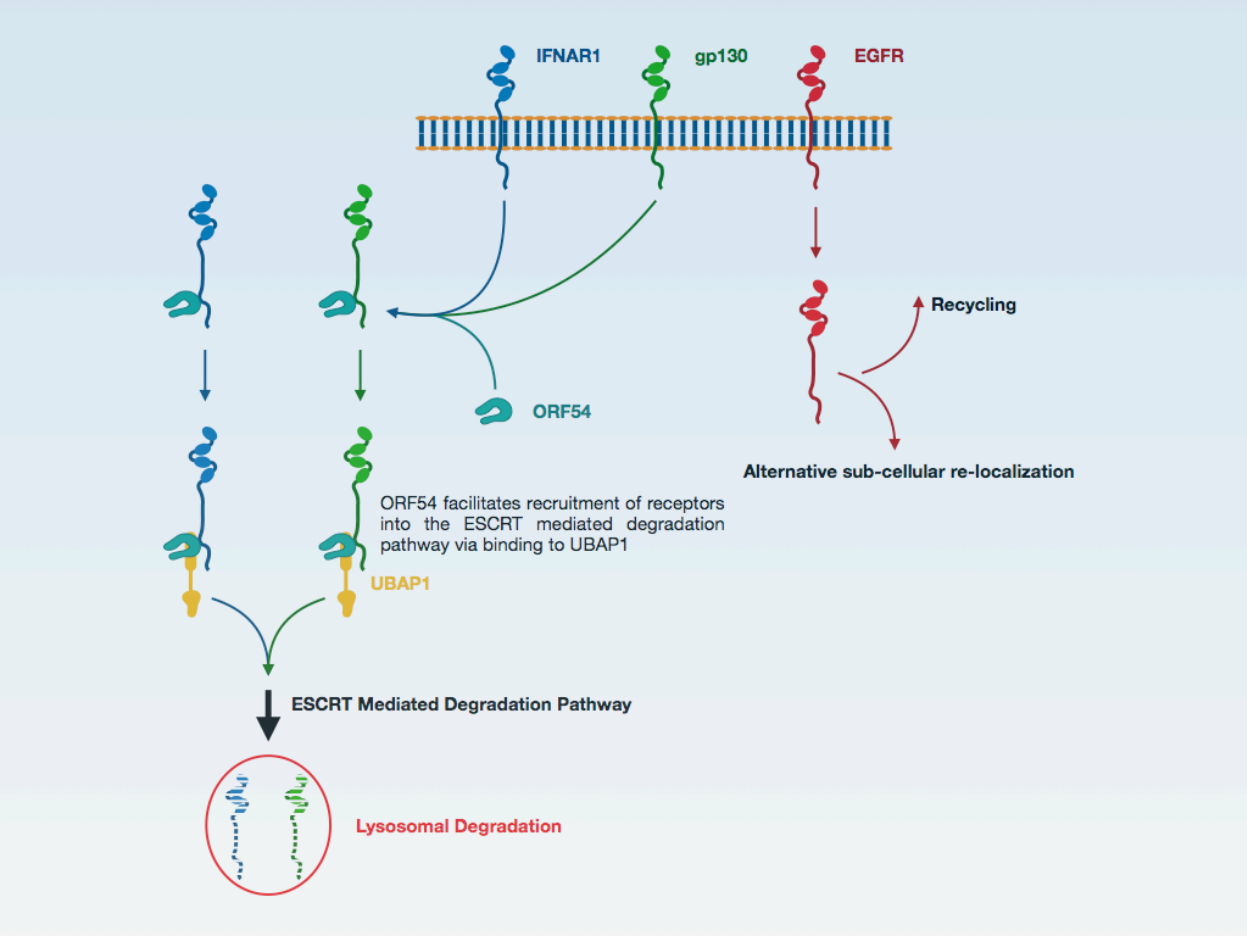


Figure 1I. *A model of ORF54 mediated cytokine receptor degradation.* The viral dUTPase encoded by ORF54 (ORF54) selectively targets cytokine receptors toward lysosomal degradation via the association of UBAP1 that facilitates the entry of targeted proteins into the ESCRT mediated degradation pathways

Materials and Methods

Cells

293T cells, HeLa, SLK, and iSLK cells were cultured in complete Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. Monocytic THP-1 and U937 cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS. To differentiate THP-1 and U937 cells into macrophages cells were incubated with RPMI and 20ng/mL of phorbol 12-myristate 13-acetate (PMA), or alternatively known as tumor-promoting agent (TPA), for 24-48hrs. Upon differentiated these cells settle and adhere, once this occurs, media is exchanged with fresh RPMI.

Immunoblotting protocol

Cells were lysed for 10 minutes on ice in lysis buffer (50 mM Tris pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM EDTA) supplemented with 1 mM PMSF, 1 mM Na3VO4, and 1 mM NaF. Lysates were then combined with 4× protein sample buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerin, 20% DTT, 0.008% Bromophenol blue), and boiled for 10 minutes before loading on an 8% polyacrylamide gel. All blots were transferred via wet transfer in transfer buffer (10% 10X Transfer Buffer [3.03% Tris.Base, 14.4% Glycine], 10% Methanol, 0.01%SDS) using activate PVDF membrane. The antibodies used in this study were rabbit anti-human phosphoSTAT1 (Cell Signaling 9167), mouse anti-FLAG M2 (Sigma F3165), rabbit anti-human IFNAR1 (Abcam, ab45172), mouse anti-IFNAR1 (H-11) (Santa Cruz Biotechnology sc-7391), rabbit anti-EGFR (Millipore 06-847), mouse anti-gp130 (E-8) (Santa Cruz Biotechnology sc-376280), mouse anti-β-actin (Sigma A5316), mouse anti-α-Tubulin Antibody (Cell Signaling 2144), rabbit anti-IFNAR2 (Novus Biologicals 31665), rabbit anti-UBAP1 (Proteintech 12385-1-AP), mouse anti-STAM2 (F-11) (Santa Cruz Biotechnology sc-365600), mouse anti-HRS (C-7) (Santa Crus

Biotechnology sc-271455), mouse anti-HHV-8 K8.1 (4A4) (Santa Cruz Antibody sc-65446). Secondary antibodies conjugated to HRP were donkey anti-rabbit IgG (GE Healthcare NA934V) and sheep anti-mouse IgG (GE Healthcare NXA931).

Co-Immunoprecipitation Assay

Cells were lysed on ice for 10 minutes and then an additional 30 minutes at 4°C using an overhead inverter with RIPA Lysis Buffer (50mM Tris.HCl, 0.5%NP-40, 150mM NaCl, 1mM EDTA) supplemented with 1mM PMSF, 1µg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Next, lysates were spun down at maximum speed at 4°C for 20 minutes to remove insoluble materials. The supernatant was decanted into a fresh falcon tube and 1-2% of volume extracted for input purposes. With exception of pre-conjugated HA (EZ view Red Anti-HA Affinity Gel E6779-1ML) and FLAG (ANTI-FLAG M2 Affinity gel A2220) affinity beads, antibody and Protein G Sepharose beads (Protein G Sepharose 4 Fast Flow GE17- 0618-01) were added to the supernatant prior to incubation at 4°C overnight shaking using an overhead inverter. Samples were spun down for 1000rpm for one minute and conjugated beads allowed to rest for two minutes on ice. The supernatant is decanted for further IP or discarded. Next beads are washed 3X using RIPA Wash Buffer (50mM Tris.HCl, 0.5% NP-40, 150mM NaCl (this may be adjusted to enhance stringency conditions), 1mM EDTA) supplemented 1mM PMSF, 1µg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Protein is eluted by boiling 15 minutes at 95°C after 4× protein Laemmli Buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerin, 20% DTT, 0.008% Bromophenol blue). Samples are loaded on an 8% polyacrylamide gel.

Biotinylation Surface Immunoprecipitation and Internalization Assay

Serum-starved cells for two hrs in serum-free DMEM supplemented with 20μg/mL of cycloheximide (CHX) to prevent surface receptor replenishing. Next, cells are removed from the incubator and placed it in warm Buffer A (0.7mM CaCl2, 0.5mM MgCl2) for five minutes and then 3X in ice-cold Buffer A for five minutes each time. Next, cells are incubated with Biotin solution (300 μg/mL of EZ- Link-Sulfo-NHS-S-S-biotin (Pierce 21331)) at 4°C on the ice, shaking very slowly. Unbound biotin is removed by three washes with ice-cold PBS followed by one was with ice-cold Buffer B (1mL of FBS in 50mL of PBS) to block remaining active sites. Next, follow protocol one for the Biotinylation Surface IP, and protocol two for the Internalization Assay.

Protocol One: Biotinylation Surface IP

Next cells are lysed using RIPA Lysis Buffer (50mM Tris.HCl, 1.0%NP-40, 200mM NaCl, 0.05% SDS, 2mM EDTA) supplemented with 1mM PMSF, 1μg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Next, lysates were spun down at maximum speed at 4°C for 20 minutes to remove insoluble materials. The supernatant was decanted into a fresh falcon tube and 1-2% of volume extracted for input purposes. The supernatant was incubated with Streptavidin Agarose beads (PierceTM High Capacity Streptavidin Agarose 20357) at 4°C overnight shaking using an overhead inverter. Samples were spun down for 1000rpm for one minute and conjugated beads allowed to rest for two minutes on ice. The supernatant is decanted for further IP or discarded. Next beads are washed 3X using RIPA Wash Buffer (50mM Tris.HCl, 0.5% NP-40, 150mM NaCl (this may be adjusted to enhance stringency conditions), 1mM EDTA) supplemented 1mM PMSF, 1μg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Protein is eluted

by boiling 15 minutes at 95°C after 4× protein Laemmli Buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerin, 20% DTT, 0.008% Bromophenol blue). Samples are loaded on an 8% polyacrylamide gel.

Protocol Two: Internalization Assay

After biotin inoculation, cells are placed in the 37°C and harvested at different time points. Surface-bound biotin is stripped by 3X incubation with 100mM MESNA (0.197g in 10mL of ddH2O) at 4°C shaking slowly. MESNA is quenched by 10 min incubation with 120mM iodoacetamide at 4°C shaking slowly. Next cells are lysed using RIPA Lysis Buffer (50mM Tris.HCl, 1.0%NP-40, 200mM NaCl, 0.05% SDS, 2mM EDTA) supplemented with 1mM PMSF, 1µg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Next, lysates were spun down at maximum speed at 4°C for 20 minutes to remove insoluble materials. The supernatant was decanted into a fresh falcon tube and 1-2% of volume extracted for input purposes. The supernatant was incubated with Streptavidin Agarose beads (PierceTM High Capacity Streptavidin Agarose 20357) at 4°C overnight shaking using an overhead inverter. Samples were spun down for 1000rpm for one minute and conjugated beads allowed to rest for two minutes on ice. The supernatant is decanted for further IP or discarded. Next beads are washed 3X using RIPA Wash Buffer (50mM Tris.HCl, 0.5% NP-40, 150mM NaCl (this may be adjusted to enhance stringency conditions), 1mM EDTA) supplemented 1mM PMSF, 1µg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Protein is eluted by boiling 15 minutes at 95°C after 4× protein Laemmli Buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerin, 20% DTT, 0.008% Bromophenol blue). Samples are loaded on an 8% polyacrylamide gel.

Ubiquitin Co-IP

Cells were lysed on ice for 10 minutes and then an additional 30 minutes at 4°C using an overhead inverter with Harsh RIPA Lysis Buffer (25mM Tris.HCl, 1.0% NP-40, 150mM NaCl, 1mM EDTA, 5% glycerol, 1% SDS) at 95°C for 5 minutes. Next, the samples are diluted with Regular RIPA Buffer (25mM Tris.HCl, 1.0% NP-40, 150mM NaCl, 1mM EDTA, 5% glycerol) supplemented 1mM PMSF, 1µg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). The lysates were spun down at maximum speed at 4°C for 20 minutes to remove insoluble materials. The supernatant was decanted into a fresh falcon tube and 1-2% of volume extracted for input purposes. The samples were then incubated with pre-conjugated HA (EZ view Red Anti-HA Affinity Gel E6779-1ML) beads to precipitated HA-tagged ubiquitin or antibody alongside Protein G Sepharose beads (Protein G Sepharose 4 Fast Flow GE17-0618-01) were added to the supernatant prior to incubation at 4°C overnight shaking using an overhead inverter. Samples were spun down for 1000rpm for one minute and conjugated beads allowed to rest for two minutes on ice. The supernatant is decanted for further IP or discarded. Next beads are washed 3X using RIPA Wash Buffer (50mM Tris.HCl, 0.5% NP-40, 150mM NaCl (this may be adjusted to enhance stringency conditions), 1mM EDTA) supplemented 1mM PMSF, 1µg/mL Aprotinin, Leupeptin, PepstatinA, 1mM Orthovanadate, and 1mM Sodium Floride (NaF). Protein is eluted by boiling 15 minutes at 95°C after 4× protein Laemmli Buffer (0.25 M Tris pH 6.8, 8% SDS, 40% glycerin, 20% DTT, 0.008% Bromophenol blue). Samples are loaded on an 8% polyacrylamide gel.

Immunofluorescence Assay

HeLa cells were plated on glass slides in 24-well plates. Next, these cells were transfected with HA-tagged ORF54 or K5 for 12-24 hrs. Cells were fixed with ice-cold methanol for five minutes

For the staining of IFNAR1 and gp130 on differentiated THP-1 cells. For co-staining of ORF54 and vesicle markers, cells were fixed using 4% PFA for 20 minutes at RT. For staining of IFNAR1 and vesicle markers in the presence of HA-tagged ORF54 and K5, we employed an IF Methanol-perm protocol. Cells were suspended in ice-cold methanol for one minute, the methanol aspirated and submerged in 4% PFA. Following fixation, samples were blocked with Blocking Buffer (1X PBS, 10% FBS, 0.3% TritonTM X-100) for one hr. Next, samples were incubated with primary antibody at 4°C overnight in Antibody Dilution Buffer (1X PBS, 1% BSA, 0.3% TritonTM X-100). Samples were rinsed with 1X PBS 3X for five minutes each time to wash off primary antibody before incubation with fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for two hrs at RT. Coverslip slides with Prolong® Gold Antifade Reagent (cell signaling 9071) and stores at 4°C for long term storage. Primary antibodies used were rabbit anti-EEA1 (C45B10) (Cell Signaling 3288), rabbit anti-Rab5 (Cell Signaling 3547), rabbit anti-Rab7 (Cell Signaling 9367), rabbit anti-Rab11 (5589), rabbit anti-LAMP1 (Cell Signaling 9091), mouse anti-EEA1 (E-8) (Santa Cruz Biotechnology sc-365652), IFNAR1 (Abcam, ab45172), mouse anti-gp130 (E-8) (Santa Cruz Biotechnology sc-376280), rabbit anti- HA antibody (Cell Signaling 3724), mouse anti-HA antibody (Sigma-Aldrich H3663-100UL), Goat anti-rabbit Alexa Fluor 488 A-11008, Goat anti-mouse Alexa Fluor 488 A-11029, Hoechst 33342 (H21492).

Real Time PCR

Total RNA was extracted via TRIzolTM (Invitrogen 15596026). The sequential precipitation of RNA was performed by homogenization with TRIzolTM followed by the addition of chloroform allowing the homogenate to separate into the clear upper aqueous layer that contains RNA. RNA is precipitated from the aqueous layer with isopropanol. The precipitated RNA is washed to

remove impurities, and then resuspended for use in downstream applications. Primers used in RT-PCR to quantify cellular transcripts are as follows:

actin: 5′-GTATCCTGACCCTGAAGTACC-3′ and 5′-TGAAGGTCTCAAACATGATCT-3′; 5’- AAGCACCTCAAAGGGCAAAAC-3’ and 5’-TCGGCCCATGTGATAGTAGAC-3’.

Plasmids

pCMV-3XFLAG-UBAP1 was a kind gift from Dr. Ting-Ting Wu at UCLA. The constructed ESCRT binding mutants and ubiquitin binding mutant show no significant change in molecular weight. The functional sites indicating the necessary mutations are available on UniProt und ID UBAP1_Human Q9NZ09 and in Table 1 and 2 below. We generated three ESCRT-I binding mutants the LDD, VPF, and a combined PE UBAP1 mutant and one ubiquitin binding mutant incorporating all three mutations into all three ubiquitin-binding domains.

Table 1.

Mutations and locations on UBAP1 clones.

Position	Mutation	Description
17-19	LDD → AAA	Abolishes ESCRT-I association
20-22	VPF → AAA	Abolishes ESCRT-I association
37	P → A	Abolishes ESCRT-I association
59	E → G	Abolishes ESCRT-I association
404	Y → A	Abolishes ubiquitin binding
439	F → A	Abolishes ubiquitin binding
472	F → A	Abolishes ubiquitin binding

Table 2.

Primer sequences for UBAP1 mutagenesis.

Primer Name	Primer Sequence
FPE1ALDDAAA	CAGTTACGCTGCTGCTGTCCCATTT
RPE1ALDDAAA	AAATGGGACAGCAGCAGCGTAACTG
FPE1AVPFAAA	CTTGATGATGCTGCAGCTAAGACAG
RPE1AVPFAAA	CTGTCTTAGCTGCAGCATCATCAAG
FPE1APA	AAGTTGGTCTAGCTATTGGCTTCTC
RPE1APA	GAGAAGCCAATAGCTAGACCAACTT
FPE1AEG	ACTTCTCTTTGGGAAAGAAAACCAT
RPE1AEG	ATGGTTTTCTTTCCCAAAGAGAAGT
FPUAMGYA	TCAACATGGGCGCCTCGTACGAGTG
RPUAMGYA	CACTCGTACGAGGCGCCCATGTTGA
FPUAKGFA	GTGAGAAGGGCGCCGACCCTCTTTT
RPUAKGFA	AAAAGAGGGTCGGCGCCCTTCTCAC
FPUAMGFA	AGGAGATGGGCGCTGAGCTGAAAGA
RPUAMGFA	TCTTTCAGCTCAGCGCCCATCTCCT
UBAP1_General_FP_BamHI	CTCTTCGGATCCATGGCTTCTAAGAAGTTG
UBAP1_General_RP_XhoI	CTCTTCCTCGAGTCAGCTGGCTCCTGCCCG

pCMVHA-KSHV ORF54 and K5, as well as the pCMV-3XFLAG-EBV BLLF3 were kind gifts from Dr. Ting-Ting Wu. The cloning of HCMV-UL72 and HSV1-UL50 homologs occurred by cloning out of the DNA of virus provided by Dr. Danyang Gong.

iSLK Reactivation System

iSLK cells are epithelial cells that express RTA and that maintain latently the viral episome of KSHV. The WT and 54S-KSHV iSLK cell lines were a kind gift of Dr. Danyang Gong, who shall receive full credit for inducing the 54S mutation and establishing the viral cell lines. We employ two mechanisms of reactivation suited to distinct purposes. To evaluate viral lytic replication in a uniform system within 72 hrs. we induce the reactivation of KSHV via treatment of 5μg/mL of doxycycline and 1mM Sodium butyrate in combination. To evaluate the gradual replication of the virus as it spreads from a minimally activated cell number, we treat the system with 1μg/mL of doxycycline alone and evaluate viral replication 5 days and 7 days post-reactivation. This second approach allows us to assess the strength of viral replication in the presence of an autocrine and paracrine type-I IFN response.

Experimental Outline

Providing simply a number of general protocols provides a critical limitation; namely, the final outline and details of experimental processes. The goal of this section is to provide some detail to critical steps of the experimental process that when observed should lead to the success of repeating experiments outlined in this dissertation.

Transfection and cell number plated

The majority of experiments in this dissertation employ the overexpression of cellular and viral plasmids. To that end, our lab prefers to employ Lipofectamine 2000 and 3000 to ensure even expression of proteins. Both of these products are well characterized and have well-established protocols. Throughout this dissertation, we expressed various amounts of DNA in the different system shown in the table below.

Table 3.

DNA amount used for transfection of ORF54 and K5.

Plate	ORF54	K5
24,12,6 well plates	1000ng/mL	500ng/mL
60mm plate	8μg/plate	4μg/plate
10cm plate	10μg/plate	6μg/plate

Cell number plated and proper plating technique

Often overlooked, it is essential to know how many cells to use for different experiments. When overexpression is the goal, maximizing cell confluency is necessary and can ensure a functional expression of protein. It is of note, however, that expression of protein has to be timed carefully to provide a consistent and reproducible phenotype. To that, I suggest experimenting with different time points to identify which is ideal. Also essential is managing cell number for various experiments. As cells are of different sizes, they will achieve confluency at different rates. After many failed tests and much time spent evaluating these factors the table below indicates the preferred conditions for the experiments of this dissertation.

Table 4.

Cell number plated for cell lines.

Cell type	Number cells plated	Experiment type
293T	$3.0x10^5$/mL	Overexpression, IP, RNA
HeLa	$5.0x10^4$/well (24-well) $1.5x10^5$/mL (12-well)	IFA (24-well plate), Flow cytometry (12-well plate)
THP-1 and U937	$5.0x10^5$/mL	Overexpression, IFA
SLK and iSLK	$1.5x10^5$/mL	KSHV reactivation experiments

Of note is the essential nature of the plating technique. When plating is aware that clumping cells will result in poor quality experimentation, to prevent this, always ensure splitting of maintenance plates occurs on time and is done evenly. Also, when plating, swirl the plate, visually validating that the cells are spread out. Then allow cells to rest at RT in the hood for 30 minutes to ensure they settle evenly before placing back into the incubator.

Chapter One: ORF54's association with ESCRT-I protein UBAP1 facilitates the selective degradation of IFNAR1.

Summary

Research approach and emphasis

The focus of this chapter is a thorough overview and interpretation of data collected from the experiments designed to evaluate the molecular mechanism of KSHV's ORF54. The majority of data presented herein is the result of biochemical and molecular biology-based assays. As such, the strength of this work is in the robustness of the phenotype observed and the orthogonality of the experimental outline. The success of scientific rigor is reproducibility and an example record that serves as the foundation of future work. To that end, the careful interpretation and evaluation of data provide a roadmap of thought as future investigations reconsidered. Each figure in this section is specifically outlined to emphasize critical discoveries. It is the goal of this chapter to successfully interpret and provide an academic context for the data to provide the reader with an understanding that enlightens the findings of this study. Throughout the experimental design, the viral E3 ligase K5 acts as a negative control. Similarly, the transmembrane receptor EGFR and the IFNAR2 subunit of the IFN receptor are unimpacted by ORF54 and thus serve to underline ORF54's selectivity in targeting surface proteins.

Overview of observations

We elucidated the molecular mechanism by which KSHV's ORF54 down-regulates IFNAR1 through the endosomal sorting complexes required for transport (ESCRT)-mediated lysosomal degradation. ORF54 targets IFNAR1 and gp130 for degradation but not EGFR or IFNAR2, demonstrating the selectivity[79]. In the case of IFNAR1, ORF54 consequently inhibits the type-I IFN responses. ORF54 interacts with ubiquitin-associated protein 1 (UBAP1), a subunit of

ESCRT-I, and this interaction is necessary for ORF54 to down-regulate IFNAR1. UBAP1 is also required for ligand-induced IFNAR1 degradation, consistent with the role of UBAP1-containing ESCRT-I in the regulation of surface receptors. Both IFN-I treatment and ORF54 enhances the association of IFNAR1 with UBAP1. While the role of UBAP1 in ESCRT-I is to bind to ubiquitinated proteins[80], two lines of evidence support that ORF54-meidated association is independent of ubiquitination. First, unlike ligand-induced association that requires functional ubiquitin associated (UBA) domains of UBAP1, ORF54 still enables the association of the UBA mutant with IFNAR1. Second, UBAP1 associates with a lysine IFNAR1 mutant devoid of ubiquitination sites in the presence of ORF54 but not upon IFN treatment[69]. Thus, ORF54 enables ubiquitin-independent interaction between IFNAR1 and UBAP1. ORF54 also interacts with IFNAR1 regardless of whether UBAP1 is present. This interaction, however, is endocytosis-dependent. Based on these results, we propose a model where ORF54 hijacks the trafficking of endocytosed IFNAR1 by recruiting IFNAR1 to UBAP1 and introducing it into the ESCRT-mediated lysosomal degradation. This molecular mechanism allows ORF54 to bypass the requirement of lysosomal ubiquitin sorting signal and re-route the un-ubiquitinated IFNAR1, preventing it from recycling back to the surface.

Introduction

Plasma membrane proteins are at the forefront of a cell to sense and respond to signals from its environment. Their expression determines the strength and duration of cellular responses to extracellular stimuli. Plasma membrane proteins undergo constitutive endocytosis. Through a highly complex endocytic network, internalized proteins are either recycled back to the cell surface or sorted towards lysosomal degradation. The predominant sorting signal for lysosomal degradation is the ubiquitin modifications within the cytoplasmic domain of endocytosed membrane proteins[80-81]. Ubiquitins are recognized by the endosomal sorting complexes required

for transport (ESCRT) apparatus comprised of a series of distinct protein complexes: ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. The first three complexes work in sequence to capture ubiquitinated proteins, forming degradative subdomains on endosomal membranes, and subsequently recruit ESCRT-III. ESCRT-III surrounds degradative subdomains and cooperates with ATPase vacuolar protein sorting 4 (Vps4) to initiate inward budding of intraluminal vesicles (ILVs) containing sequestered ubiquitinated proteins[83]. Through this ESCRT-mediated sorting process, numerous ILVs are generated to form a multivescular body (MVB), which subsequently fuse with lysosomes for degradation. The half-life and surface level of a plasma membrane protein, such as a cytokine receptor, is determined by the rates of internalization, recycling, and degradation. Therefore, cellular responses to extracellular environment stimuli can be regulated by endocytic trafficking of the responding receptor to modulate its surface expression. Hijacking the trafficking of plasma membrane proteins that impart anti-viral responses to degradation allows viruses to achieve optimal replication inside a cell surrounded by a hostile environment.

Overcoming the type-I IFN response is a significant step to the survival of the virus. IFNα/β signals through a ubiquitously expressed heterodimeric receptor, IFNAR, comprised of IFNAR1 and IFNAR2. Therefore, almost every aspect of this anti-viral response is targeted by viruses, including surface expression of IFNAR1[84]. IFNAR1 is targeted by several viruses. The major mechanism of IFNAR1 down-regulation determined so far has been due to an indirect consequence of signaling pathways induced by viral proteins or infection, resulting in IFNAR1 phosphorylation that recruits SCF^{HOS} E3 ligase, which ubiquitinates IFNAR1 [69,71]. Ubiquitination of IFNAR1 promotes its internalization and endocytic sorting towards lysosomal degradation[69-70]. These virus-induced signaling pathways include unfolded protein pathway activated PERK[85] and the pattern recognition pathway activated p38 kinase[86].

Previously we identified ORF54, a functional dUTPase, as an antagonist of IFNα/β. ORF54 down-regulates the protein level of IFNAR1, thereby diminishing the ability of infected cells to respond to IFNα/β. Of note in the present study are KSHV ORFs that encode ubiquitin ligases and down-regulate surface molecules critical for immune activation and communication. The viral ubiquitin ligases, K3 and K5, act on the cytoplasmic domains of multiple surface proteins, such as MHC class I, to increase their endocytosis and cause them to be sorted toward lysosomal degradation[87]. Interestingly, both K3 and K5 downregulate the IFNγ receptor, but they have no effect on IFNAR1[55]. Except for MHV-68, which encodes a K3 homologue that mediates proteosomal degradation[87], no other herpesvirus has been found to encode similar E3 ligases that ubiquitinate membrane proteins. ORF54 does not have any recognizable E3 ligase domain, such as RING or HECT, indicating a different mechanism from K3 or K5. Therefore, it remains to be determined how ORF54 regulates surface expression of IFNAR1 and possibly other plasma membrane proteins as well[69].

The ESCRT machinery participates in many other cellular processes as well as viral budding in addition to the well-recognized role in the formation of MVB to deliver ubiquitinated cargoes to lysosomes[88]. Mammalian ESCRT-I exits in multiple forms due to variants of its subunits, and may contribute to regulating diverse activities of ESCRT. Among four subunits of ESCRT-I, TSG101 and VPS28 are constant while VPS37 and MVB12 have multiple variants. Ubiquitin-associated protein 1 (UBAP1), a recently identified MVB12-like subunit, defines a specific form of ESCRT-I that is required for epidermal growth factor receptor (EGFR) down-regulation[80] as well as for K5-mediated degradation of anti-viral surface proteins, but not for viral budding[90]. UBAP1 was reported to be a risk factor for familiar frontotemporal lobar dementia (FTLD)[91] and its expression was found reduced in nasopharyngeal carcinoma[92]. All three MVB12-like variants identified so far share a UBAP1-MVB12-associated (UMA) domain that mediates their

incorporation into ESCRT-I [80,90]. Different from the other two MVB12-like subunits, MVB12A and B, UBAP1 does not have a membrane-targeting MABP domain but has three ubiquitin-associated (UBA) domains, also referred to as a solenoid of overlapping UBA (SOUBA) domain [80,90]. The SOUBA domain of UBAP1 is responsible for ubiquitin binding and required for MVB sorting of EGFR.

This chapter describe the studies of the molecular mechanism by which KSHV's ORF54 down-regulates IFNAR1 as well as gp130 through ESCRT-mediated lysosomal degradation.

Results

ORF54 selectively targets cytokine receptors for degradation and limits the induction of the type-I IFN response.

Here we investigated the ability of KSHV's ORF54 to degrade IFNAR1 and gp130. 293T cells were transfected with HA-tagged protein expression plasmids of ORF54 and K5. As can be observed, ORF54 significantly reduces both IFNAR1 and gp130 compared to K5 (Fig. 1-1a). In contrast, IFNAR2 and EGFR remain largely unaffected by ORF54. To examine whether this phenotype is limited to 293T cells, we repeated the experiment in a monocytic cell line, U937, differentiated into macrophages. After differentiation, the protein expression plasmids were transfected. The western blots confirmed the down-regulation of IFNAR1 in the presence of ORF54 (Fig. 3-1a). Another viral protein encoded by ORF10 that shares a dUTPase-related domain with ORF54 also reduces IFNAR1. In subsequent experiments, we determined that ORF10 employs a different mechanism from ORF54 (Fig. 1-4b)

To evaluate the functional impact of ORF54 on the type-I interferon response, we measured the induction of interferon-stimulated gene 54 (ISG54) using RT-qPCR (Fig. 1-1b). The loss of ISG54 induction in the presence of ORF54 supports that ORF54 functionally antagonizes the

type-I interferon response by inducing the degradation of IFNAR1. We did not evaluate the induction of the IL-6 response but believe that the signaling through the IL-6 receptor is limited due to the loss of gp130.

ORF54 post-translationally regulates IFNAR1.

Previous work on MHV-68's ORF54 determined that ORF54 does not affect the total transcript level of IFNAR1. Here we determined the impact of KSHV's ORF54 on the protein half-life. The experiments were performed in 293T cells treated with a translation inhibitor, cycloheximide (CHX), and quantify the IFNAR1's protein level at different time points after CHX treatment. The half-life of IFNAR1 in ORF54-transfected cells is significantly shorter in cells transfected with K5. This indicates that ORF54 functions post-translationally to down-regulate IFNAR1. During this four-hour time course, EGFR remains unchanged in both transfections, indicating a longer half-life than IFNAR1.

ORF54 reduces the abundance of cytokine receptors at the cell surface.

Both IFNAR1 and gp130 are transmembrane receptors that induces signaling upon ligand binding. The loss of ISG induction upon IFNα treatment indicates that not only the total protein level but also the surface expression of IFNAR1 and gp130 is affected by ORF54. We performed surface protein-specific IP experiments and determined the amount of IFNAR1 and gp130 by western blots (Fig. 1-1d). The specificity of surface IPs is attested by the absence of tubulin. IFNα treatment is known to induce IFNAR1 internalization and thus, was included as a control. Both ORF54 and IFNα reduce the surface expression of IFNAR1 and gp130 compared to the K5 control without affecting EGFR and IFNAR2. It was somewhat suprising for us to see gp130 affected by IFNα, but this has been observed perviously by others[93-94].

Because IFNAR1 expression in 293T cells is too low to be reliably detected by flow ctyometry, we used HeLa cells that express ORF54 upon induction with tetracycline. We were able to detect IFNAR1 by flow cytometry and showed that the percentage of IFNAR1-positive cells is signficantly reduced upon induction of ORF54 expressing (Fig. 1-1e). By IFA we also observed a loss of IFNAR1 and gp130 at the cell surface of macrophages differentiated from THP-1 cells (Fig. 1-1f). Taken together, we demonstrated that ORF54 efficiently reduces the surface expression of IFNAR1 and gp130, thus limiting availavle cytokine receptors to engage ligands and initiate signaling. We focus on futher charcterizing IFNAR1 in the following studies.

ORF54 employs the ESCRT pathway to facilitate lysosomal degradation of IFNAR1.

Cells employ proteasomes and lysosomes as the two primary mechanisms to degrade proteins [95-96]. To identify which of these two mechanisms employed by ORF54, we applied the small molecule inhibitors. We examined IFNAR1 expression in the cells treated with either MG132, an inhibitor of 26S proteasome complex, or chloroquine (CQ), an inhibitor of lysosomal enzymes by preventing endosomal acidification. CQ rescues IFNAR1 expression from ORF54-mediated degradation (Fig. 1-2a) while MG132 did not. Thus, ORF54 enhances lysosomal degradation of IFNAR1. We noticed that CQ treatment increases expression of IFNAR2 and EGFR as well compared to the vehicle control. This indicates that a fraction of surface proteins undergoes constitutive lysosomal degradation.

We also observed that surface expression of IFNAR1 is also rescued by CQ (Fig. 1-2b). Accordingly, IFNα-induced phosphor-STAT1 is restored in ORF54-transfected cells treated with CQ (Fig. 1-2c). Furthermore, we also demonstrated that ORF54 transfection alone does not induce STAT1 phosphorylation. CQ prevents endosomal acidification, which can reduce overall endosomal trafficking. It remains to be determined whether CQ slows down IFNAR1

internalization or allows IFNAR1 to escape from routing to lysosomes and recycle back to the surface.

The ESCRT machinery is critical for trafficking of endocytosed proteins to lysosomes. At the final membrane scission step, ESCRT-III recruits ATPase Vps4 to drive inward budding of ILVs. Vps4 is also responsible for disassembly of ESCRT-III and allowing it to recycle for subsequent use. The dominant negative of Vps4 (Vps4-DN) lacking ATPase activity was thus developed to inhibit the ESCRT machinery [97-100]. We employed this DN mutant to complement the chemical approach of using CQ. Cells were co-transfected with combinations of Vps4-DN or Vps4-WT and ORF54 or K5. In the presence of Vps4-DN (DN), IFNAR1 expression is restored and comparable between K5- and ORF54-transfected cells. Much like treatments with CQ, VPS4-DN also rescues IFNAR1 expression at the cell surface and reinstate IFNα-induced phosphor-STAT1 in ORF54-transfected cells (Fig. 1-2e & f). These results support the hypothesis that IFNAR1 is selectively shuttled towards lysosomes in the presence of ORF54.

ORF54 does not enhance the endocytosis of IFNAR1

We next determined whether ORF54 targets the endocytosed IFNAR1 or the newly synthesized protein on its way to the cell surface. To distinguish these two possibilities, an inhibitor of clathrin-mediated endocytosis, chlorpromazine (CPZ), was used. As shown in Fig. 1-2g, stopping endocytosis by CPZ is able to abolish the effect of ORF54 on IFNAR1, supporting that it is the endocytosed species that ORF54 targets.

As mentioned in the beginning, ligand binding to a surface receptor accelerates receptor internalization and its sorting to degradation, leading to down-regulation of surface expression of the receptor. In the case of IFNAR1, upon IFN binding, IFNAR1 is phosphorylated by its associated kinases, and acts as the docking site to recruit SCF^{HOS} Ring E3 ligase complex, which

subsequently ubiquitinates IFNAR[68-72]. Ubiquitination of IFNAR1 increases its internalization and endocytic sorting towards lysosomal degradation[68-72]. This ligand- and ubiquitin-dependent endocytosis and degradation represents a major mechanism to terminate the signaling from cell surface receptors. It has been shown that viruses hijack this regulation to down-regulate IFNAR1. Infection or viral proteins activate signaling pathways that lead to phosphorylation and ubiquitination of IFNAR1, thereby inducing its internalization and lysosomal degradation.

To determine whether ORF54 increases the endocytosis of IFNAR1, we performed internalization assays using modified surface IPs. After conjugating surface proteins with biotin on ice, cells were incubated at 37°C to allow for endocytosis. At various times, biotinylated surface proteins were stripped and internalized biotinylated proteins were purified by streptavidin beads for western blots. Clearly, both IFNAR1 and EGFR undergo internalization without adding the ligands (Fig. 1-2h). There seems to be a faster internalization rate for EGFR than IFNAR1. Importantly, the internalization rate of IFNAR1 does not differ significantly between ORF54- and K5-transfected cells. Unlike other known viral mechanisms of IFNAR1 down-regulation, ORF54 does not induce IFNAR1 phosphorylation as IFNα treatment (Fig. 1-2i). Taken together, these results indicate that ORF54 employs a mechanism distinct from activation of signaling pathways to alter the fate of surface IFNAR1.

Work by the Fuchs lab has shown that IFNAR1-associated Tyk2 blocks a critical Tyr-422 amino acid side chain on the cytoplasmic domain of IFNAR1, thereby preventing the AP2 complex from binding and initiating clathrin-coated endocytosis[72-74]. When we probed for Tyk2 in the IFNAR1 IP samples, there was much less Tyk2 association in cells transfected with ORF54 and those treated with IFNα compared to K5-transfected cells. Because ORF54 does not affect IFNAR1 internalization, it is unlikely that it knocks off Tyk2, which would result in endocytosis

of IFNAR1. These results suggest that ORF54 does not influence IFNAR1 endocytosis via biochemical modifications. The implication of this observation remains to be explored.

ORF54 induces IFNAR1's accumulation in endosomal compartments

We performed a series of IFA to examine the localization of ORF54 and IFNAR1. We employed early endosome markers Rab5 and EEA1, the late endosome marker Rab7, the recycling endosome marker Rab11 as well as the lysosomal marker LAMP1. ORF54 is ubiquitously expressed in the cytoplasm and co-localizes with Rab5, EEA1 as well as Rab7 but not Rab11 or LAMP1 (Fig. 1-3a). This observation suggests that ORF54 functions at the endosomal compartments to alter the trafficking of endocytosed IFNAR1. This hypothesis is supported further by evidence that *the* loss-of-function mutants of ORF54 lose the ability to localize in the early and late endosome. Instead, the N72A and D160A mutants are found exclusively in the nucleus, while the Y267A mutant remain cytosolic, but is not associated with either endosomal compartment.

Based on the results that ORF54 does not induce IFNAR1 internalization and that ORF54 targets the endocytosed IFNAR1, we hypothesized that ORF54 facilitates the entry of IFNAR1 into ESCRT-mediated lysosomal degradation. This would lead to increased localization of IFNAR1 at endosomal compartment in cells expressing ORF54. Indeed, we observed a significant co-localization of IFNAR1 with EEA1 (Fig. 1-3b).

ORF54's association with UBAP1 is critical for the degradation of IFNAR1

Previous work identified the ubiquitin-binding protein 1 (UBAP1) as a direct interaction partner of ORF54 using a Yeast-2-Hybrid (Y2H) screen [68]. We validated the Y2H result in 293T cells via co-IPs (Fig. 1-4a). UBAP1 was recently identified as a subunit of ESCRT-I. UBAP1 is a ubiquitin-binding protein involved in binding and sequestering of ubiquitinated cargo proteins

onto ESCRT-I[80]. We hypothesized that the interaction with UBAP1 is required for ORF54 to down-regulate IFNAR1. To test the hypothesis, UBAP1 expression was knocked down by siRNA and indeed, IFNAR1 was restored in ORF54-transfected cells (Fig. 1-4b). However, UBAP1 knockdown did not alter ORF10-mediated down-regulation, indicating a different mechanism employed by ORF10.

Investigating the role of UBAP1 further, we attempted to generate the loss-of-interaction mutants of ORF54. We introduced individual alanine mutations at six residues conserved among KSHV, EBV and MHV-68, based on the assumption that the interaction with UBAP1 is critical for gammaherpesivuses and therefore, would be conserved. The attempt was successful as three ORF54 mutants that lost their interactions with UBAP1 (Fig. 1-4c) also failed to down-regulate IFNAR1 (Fig. 1-4d). This result further cements the critical role of UBAP1 in ORF54-mediated IFNAR1 down-regulation.

ORF54 enhances the association between UBAP1 and IFNAR1[1]

UBAP1 is thought to function in ESCRT-I to accept ubiquitinated proteins from ESCRT-0. UBAP1 is found in the early endosome and gradually increases to peak abundance in the late endosome. The IFA data showed that ORF54 localizes in the endosomal compartments and that IFNAR1 accumulates in the endosome when ORF54 is expressed. These observations led to the hypothesis that ORF54 enhances the association between IFNAR1 and UBAP1 to facilitate the entry of endocytosed IFNAR1 into the ESCRT-mediated lysosomal degradation pathway.

The association between IFNAR1 and UBAP1 was investigated by co-IP. IFNα and IFNβ were included for comparisons. Ligand binding induces the ubiquitination and degradation of IFNAR1 and as such, IFN treatment should also promote the association between IFNAR1 and UBAP1 as well. This prediction was correct as shown in Fig. 1-4e. Importantly, we determined that ORF54

also enhances the association between IFNAR1 and UBAP1. Such an enhancement is specific for IFNAR1 and not observed for EGFR or IFNAR2. These observations suggest that UBAP1 functions in recruiting IFNAR1 to the ESCRT machinery and facilitated down-regulation of the receptor subunit in the presence of ORF54.

ORF54 bypasses the requirement of ubiquitins for IFNAR1 association with UBAP1[1]
Because UBAP1 is responsible for binding ubiquitinated proteins, we predicted that the association between UBAP1 and IFNAR1 depends on ubiquitins. To test this, we employed an IFNAR1 lysine mutant devoid of ubiquitination [72]. (Fig. 3-1d). This mutant has lysine 501, 525 and 526 mutated to alanines and is resistant to degradation upon IFN treatment or by ORF54 expression (Fig. 3-1A). Cells were transfected with the WT or lysine mutant of FLAG-tagged IFNAR1 and co-IP was performed with the anti-UBAP1 antibodies. As predicted, IFNα induces association of UBAP1 only with WT but not the lysine mutant. To our surprise, ORF54 mediates the association with both WT and the lysine mutant (Fig. 1-4f). It is, however, possible that in the presence of ORF54 alternative lysine residues on the cytoplasmic domain of IFNAR1 are ubiquitinated. Therefore, to further examine the ubiquitin-independent interaction between UBAP1 and IFNAR1, we generated an UBAP1 mutant that have detrimental mutations in its three ubiquitin-associated (UBA) domains to abolish the ubiquitin-binding ability [80,89]. We reasoned that if ORF54 enables ubiquitin-independent association between UBAP1 and IFNAR1, the ubiquitin-binding mutant would retain the ability to associate with IFNAR1. We repeated co-IP experiments using the FLAG-tagged WT and ubiquitin-binding mutant. Indeed, in the presence of ORF54, the association with IFNAR1 occurs for both WT and the mutant (Fig. 1-4g). As expected, upon IFNα treatment IFNAR1 only associates with WT and not the mutant. The mutant still interacts with VPS37A, a known interacting partner of UBAP1 in the ESCRT-I complex.

To confirm the functional significance of ubiquitin-independent association between UBAP1 and IFNAR1, we reconstituted cells deficient in UBAP1 with different UBAP1-expression constructs. We employed CRISPRi technology with gRNA targeting 5'untranslated region of UBAP1 to repress its expression. Prior to transfection of UBAP1 expression constructs, cells were harvested for western analysis to confirm the knockdown and ORF54 expression indicated by probing with the anti-HA antibody (Fig. 1-4h). In addition to the ubiquitin-binding mutant, we also generated multiple UBAP1 mutants that cannot be assembled into ESCRT-I due to the mutations in the UBM domain, UBM(LDD), UBM(VPF), and UBM(PE). Both WT and the ubiquitin-binding mutant were able to reconstitute ORF54-mediated IFNAR1 down-regulation in UBAP1-deficient cells, but not the UBM mutants that cannot be incorporated into the ESCRT-I complex. As shown in Fig. 1-4g, the ubiquitin-binding mutant retains the ability to interact with VPS37A, a known interacting partner of UBAP1 in the ESCRT-I complex. On the other hand, only WT and none of the mutants can restore IFN-mediated IFNAR1 down-regulation (Fig. 3S).

Therefore, the ability of UBAP1 to associate with ESCRT-I, but not its ubiquitin-binding ability, is required for mediating IFNAR1 down-regulation in the presence of ORF54. These observations support a model in which via interacting with UBAP1 ORF54 introduces IFNAR1 into the ESCRT-mediated lysosomal degradation pathway without the need of IFNAR1 ubiquitination, bypassing a critical endogenous regulatory step.

ORF54 directs endocytosed IFNAR1 towards lysosomal degradation

The associations we observed between ORF54 and UBAP1 as well as between UBAP1 and IFNAR in the presence of ORF54 led us to hypothesize that ORF54 serves as an adaptor to recruit IFNAR1 to UBAP1. This is further supported by the similar endosomal localization shared between ORF54 and IFNAR1. Indeed, we detected the interaction between ORF54 and

IFNAR1 by co-IP (Fig. 1-5a). This interaction was not affected by CQ but completely abolished by CPZ that inhibits clathrin-mediated endocytosis, indicating that IFNAR1 becomes available for ORF54 to grab only after internalization. In addition, CPZ also prevented the association of IFNAR1 with UBAP1 induced by IFN treatment or ORF54 expression. Therefore, we concluded that UBAP1 or ORF54 does not interact with the surface-bound IFNAR1 and the three proteins become associated together most likely at endosomal compartments after IFNAR1 endocytoses.

The association between IFNAR1 and UBAP1 depends on the presence of ORF54. We next asked whether the interaction between IFNAR1 and ORF54 depends on UBAP1. To test this, we utilized the ORF54 mutants that fail to associate with UBAP1 (Fig. 1-4d). The co-IP result showed that these UBAP1-binding mutants still interact with IFNAR1 (Fig. 1-5b), supporting an UBAP1-independent association between ORF54 and IFNAR. This is further supported by the observation that the ORF54-IFNAR1 association is not affected by UBAP1 knockdown (Fig. 1-5c). Taken together, we propose that after IFNAR1 internalizes, ORF54 associates with IFNAR1 and funnel it into the ESCRT machinery through interacting with UBAP1, thereby preventing it from recycling back to the surface.

Generally, ESCRT-0 initiates ESCRT-mediated lysosomal degradation. ESCRT-0 localizes on the endosome through interacting with a specific phospholipid, phosphatidylinositol-3-phosphate (PI3P) on endosomes. ESCRT-0 seizes ubiquitinated proteins and recruits ESCRT-I. We hypothesized that ORF54 bypasses ESCRT-0 recruitment and ubiquitination, the critical lysosomal soring signal, to funnel IFNAR1 to lysosomes by bridging the interaction between IFNAR1 and ESCRT-I. To test this, we knocked down HRS and STAM2, the two key components required for the function of ESCRT-0[100]. Loss of HRS prevents the binding of ubiquitinated cargo in the early endosome, while the KD of STAM2 prevents the formation of

the ESCRT-0 complex. We showed that ORF54 continues to degrade IFNAR1 even when HRS or STAM2 expression is knocked down. This supports our model that ORF54 funnel endocytosed IFNAR1, regardless of its ubiquitination status, into the ESCRT-mediated lysosomal degradation pathway by interacting with UBAP1, the key component of ESCRT-I.

Discussion

The Endosomal Sorting Complex Required for Transport.

The function of the ESCRT machinery in the regulation, translocation, and cell mitosis has been well established. ESCRT is essential to viral entry and budding, making it key to the health of the viral life. Of note to the mechanism of ORF54 is the role of UBAP1 in the ESCRT complex. Of ESRCT-I complexes, only ~10% are positive for UBAP1. Uniquely, these UBAP1 positive ESCRT-I complexes are responsible for shuttling ubiquitinated cargo towards lysosomal degradation. This observation places UBAP1 into the function of an entry point protein into the ESCRT mediated degradative pathway. It is our opinion that it is UBAP1's central role in this mechanism that marked it for viral manipulation resulting in the targeting of IFNAR1 and other cytokine receptors. Therefore, UBAP1's essential role in the MVB/lysosome degradation pathway enables ORF54 to reroute the trafficking of surface molecules, such as IFNAR1, to lysosomal degradation.

ORF54 provides the virus with a mechanism that bypasses critical cellular regulatory functions that facilitate the routing of endocytosed IFNAR1 to the MVB pathway. ORF54 mitigates the need for ubiquitin to initiate the entry of IFNAR1 into the ESCRT mediated degradation pathway via UBAP1. The ubiquitination of the cytoplasmic domain of transmembrane proteins regulates sorting via ESCRT through the MVB pathway[80,81]. Ubiquitinated membrane proteins are captured by the ESCRT-0 complex that consists of multiple subunits that contain ubiquitin-

binding domains (UBDs). Alternatively, structurally related ubiquitin-binding domain (UBD)-containing proteins may also act as an alternative ESCRT-0 (Alt-0) to capture ubiquitinated proteins. The function of UBAP1 as part of the ESCRT-I complex is to receive ubiquitinated proteins from ESCRT-0 and transfer cargo to the ESCRT-II complex. Without ubiquitin modifications, endocytosed membrane proteins are not actively sorted to the MVB and thus, are recycled back to the surface. ORF54 is not localized on the surface and its interaction with IFNAR1 is dependent on endocytosis (Fig). While ORF54 function analogously to ESCRT-0, ORF54 does not have any recognizable UBD[109].

Further emphasizing this observation is ORF54's ability to retain the association with the KR IFNAR1 mutant devoid of ubiquitination, providing evidence to the conclusion that ORF54 does not bind ubiquitin. Moreover, the ORF54-mediated association between IFNAR1 and UBAP1 is also independent of IFNAR1's ubiquitination status as evidenced by two vital experimental findings. The UBAP1UBM mutant and the IFNAR1 KR mutant both maintain their association with their WT counterparts in the presence of ORF54 (Fig). In addition, the ubiquitin-binding UBAP1 mutant is still able to reconstitute ORF54-mediated IFNAR1 degradation in cells depleted of endogenous UBAP1 (Fig). In contrast, ligand-induced IFNAR1 degradation depends on the ubiquitin-binding ability of UBAP1. Taken together, our data support a model whereby the ubiquitin-independent interactions enabled by ORF54 allows the virus to reroute endocytosed IFNAR1 not designated for degradation to enter the ESCRT/MVB pathway. Required to both viral and ligand-based mechanisms is UBAP1's association with the ESCRT-I complex, as overexpression of various ESCRT-I binding mutants does not reconstitute IFNAR1's degradation. These observations imply that ORF54 bypasses essential protein modifications and biochemical alterations to target IFNAR1, lowering the requirements to degrade the receptor subunit.

Endogenously ubiquitination is essential for the entry of internalized membrane proteins into the ESCRT machinery as well as subsequent recognition and association with UBD-containing proteins of the machinery. Therefore, it is not surprising that ORF54 does not completely eliminate the need of IFNAR1 ubiquitination to ensure lysosomal degradation of the receptor species. We noted that the KR mutant of IFNAR, while still interacting with UBAP1 and ORF54, is resistant to ORF54-mediated lysosomal degradation. This suggests to us that un-ubiquitinated IFNAR1 after being recruited to ESCRT-I by ORF54 still needs to undergo ubiquitination, most like via E3 ligases associated with the ESCRT machinery, for successful sorting to MVB/lysosome.

ORF54 induced endosomal sorting of selectively targeted cytokine receptors.[1]

Another salient aspect of ORF54-mediated receptor down-regulation is its selectivity. Although UBAP1 is essential for ligand-induced down-regulation of EGFR and IFNAR1 (Fig), ORF54 selectively targets IFNAR1 and not EGFR. In addition to IFNAR1, other surface proteins, such as gp130, are also targeted by ORF54 [67]. Because ORF54 interacts with internalized IFNAR1, we hypothesize that the specific molecular interactions between ORF54 and its targets determine the selectivity of ORF54's functions.

Surface molecules undergo basal endocytosis as a result of constitutive internalization of the plasma membrane. After internalization, un-ubiquitinated signaling receptors, not actively sorted to MVB, are recycled back to the plasma membrane by the bulk membrane flow process[80-81,109]. Increasing evidence has indicated that recycling can be governed by sequence-specific interactions between adaptor proteins and sorting motifs within the cytoplasmic tail of membrane proteins.

Endocytic recycling of IFNAR1 or gp130 is mostly unknown. It remains to be determined whether ORF54 serves as an adaptor protein and recognize specific motifs within cytoplasmic tails of its targets. Alternatively, ORF54 might interact with a previously unknown cellular adaptor protein that regulates endocytic trafficking of a subset of surface molecules.

Combining our observations with the known mechanistic insight of IFNAR1 regulation, we believe to have uncovered a novel mechanism of viral type-I IFN antagonism, suggesting that herpesviral dUTPases can selectively target constitutively endocytosed surface moleculs, such as cytokine receptors and bypass vital regulatory steps that mark membrane proteins for degradation.

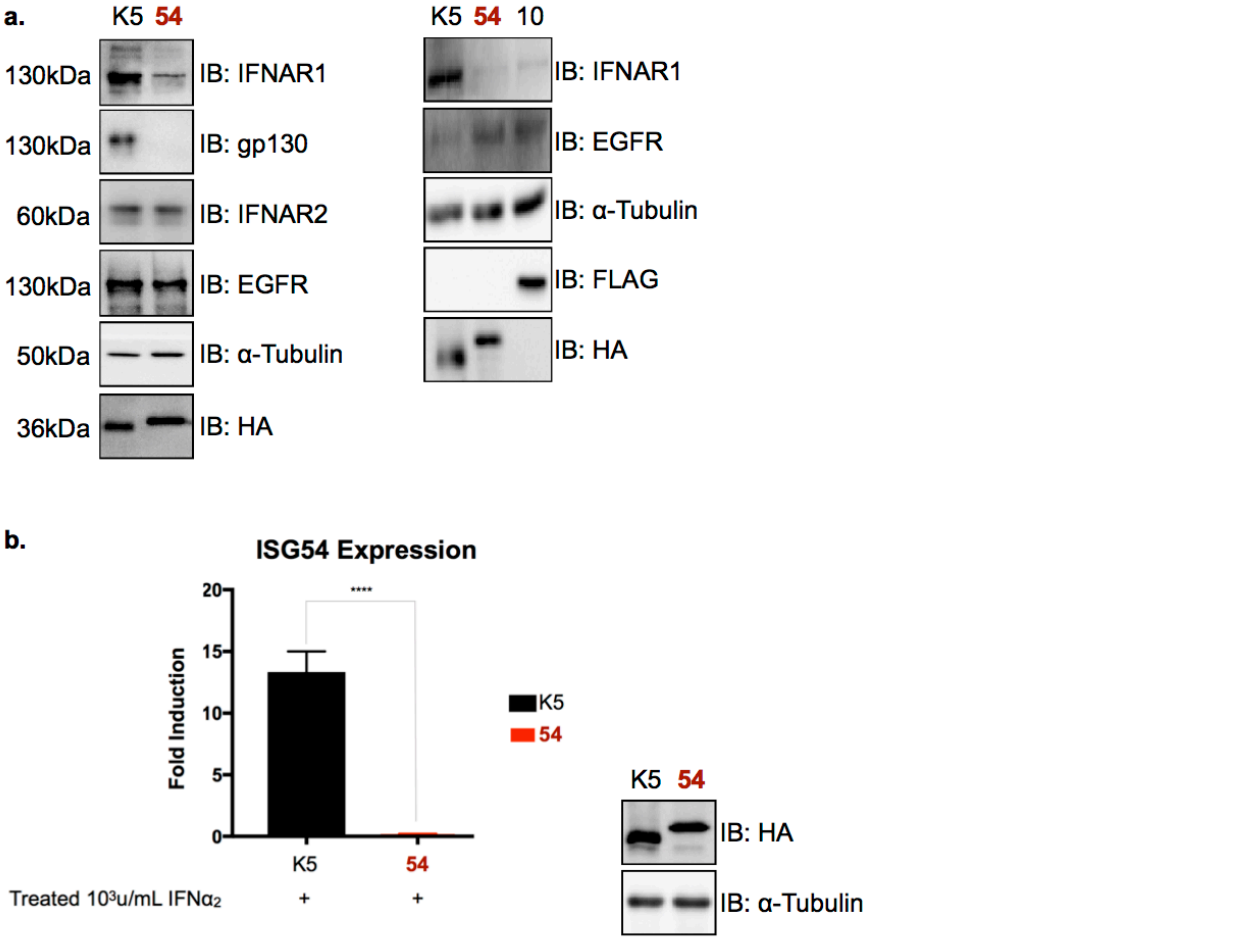
Endogenously, IFNAR1 is a regularly endocytosed and recycled, limiting the amount of receptor available on the cell surface. This cellular function is essential to ensure a limited and appropriate type-I interferon signal. A ligand- and ubiquitin-independent mechanism of IFNAR1 endocytosis identifies Tyk2 as an essential protein regulating the receptor subunit's cell surface half-life. Dissociation of Tyk2 during the inactivated receptor state exposes the Tyr-422 amino acid side chain, endorsing the binding of the AP2 complex and initiating Clathrin-Coated Endocytosis of IFNAR1. Our data indicate that less Tyk2 is less associated with IFNAR1 in the presence of ORF54 Fig. Furthermore, there we observed a notable absence of phosphorylated IFNAR1 in the presence of the viral dUTPase, contrary to the observation made during ligand-induced internalization of IFNAR1. In combination, this suggests that ORF54 binds an unmodified species of IFNAR1 thereby preventing its recycling to the cell membrane, and supports the association with UBAP1 to guarantee entry to the ESCRT-mediated degradation pathway.

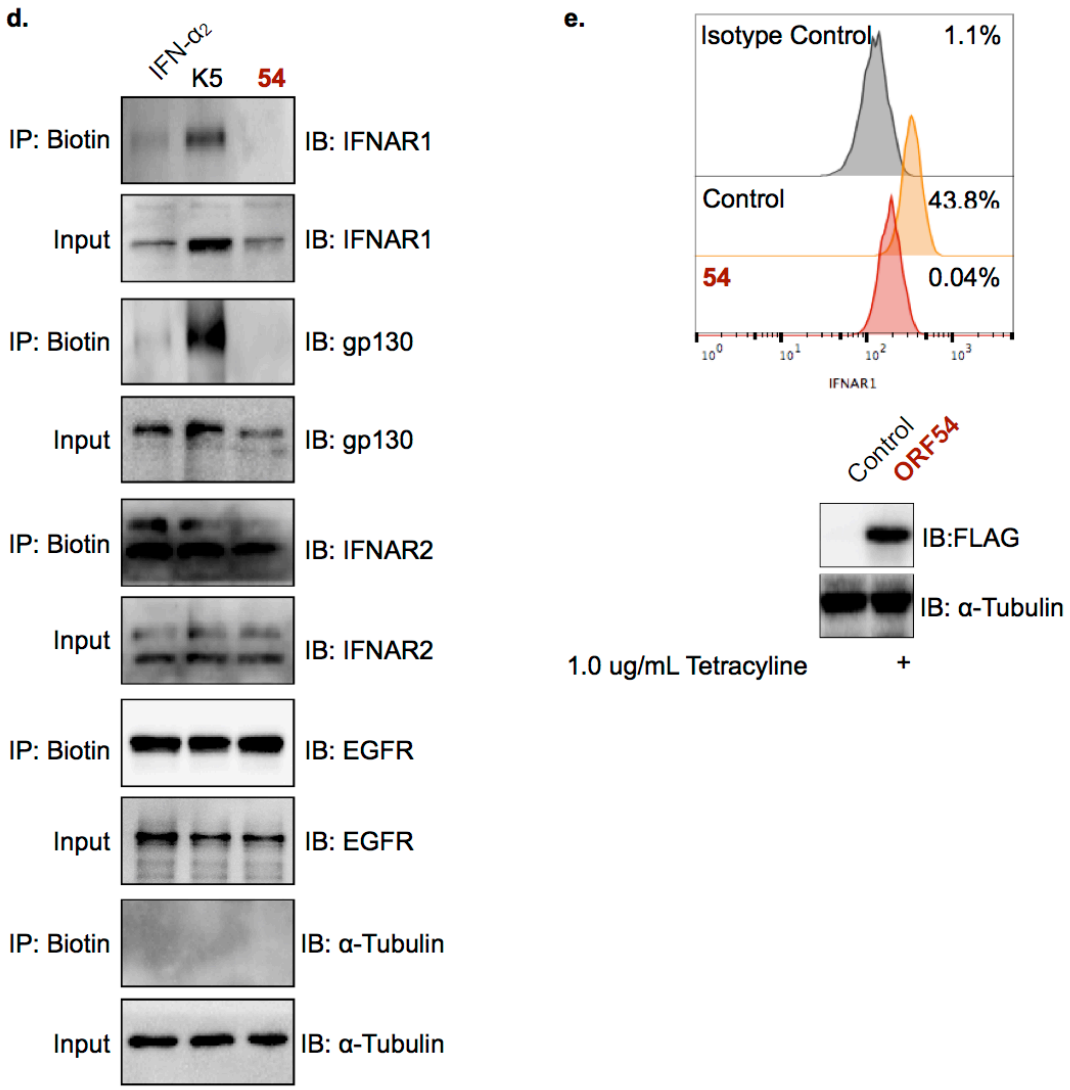
Conclusion and future perspectives.

Our experimental results lead us to conclude that ORF54 targets constitutively endocytosed IFNAR1. Much remains to be investigated about ORF54 and its manipulation of selective receptor trafficking. How does ORF54 select its targets? Does ORF54 serve as an adaptor protein and recognize specific motifs within cytoplasmic tails of cell surface proteins? Alternatively, does ORF54 interact with a previously unknown cellular adaptor protein that regulates endocytic trafficking of a subset of cytokine receptors? What is the breadth of ORF54 effect?

Since endocytic recycling of IFNAR1 or gp130 is mostly unknown ORF54 provides an opportunity to enhance our understanding of how the surface expression of these cytokine receptors can be regulated. Such insight may provide useful knowledge to improve the therapeutic efficacy of cytokines or to mitigate associated cytokine diseases.

Transfection studies allow detailed characterization of biochemical events and molecular mechanisms by which ORF54 down-regulates cell surface receptors. It is, however, essential to study the ORF54's effect in the context of viral replication. The following chapter will explore ORF54's function in the context of infection.


a.
K5 54
130kDa
IB: IFNAR1
130kDa
IB: gp130
60kDa
IB: IFNAR2
130kDa
IB: EGFR
50kDa
IB: α-Tubulin
36kDa
IB: HA
K5 54 10
IB: IFNAR1
IB: EGFR
IB: α-Tubulin
IB: FLAG
IB: HA
b.
ISG54 Expression
****
Fold Induction
20
15
10
5
0
K5
54
Treated 10³u/mL IFNα₂
+
+
K5 54
IB: HA
IB: α-Tubulin
c.
K5
54
0 0.5 2.5 4 0 0.5 2.5 4 Hrs.
IB: IFNAR1
IB: EGFR
IB: α-Tubulin
IB: HA
IFNAR1 Half-Life
Relative IFNAR1 Expression
1.5
1.0
0.5
0.0
0 1 2 3 4 5
Hours
KSHV-HAK5
KSHV-HAORF54


d.
IFN-α2
K5
54
IP: Biotin
IB: IFNAR1
Input
IB: IFNAR1
IP: Biotin
IB: gp130
Input
IB: gp130
IP: Biotin
IB: IFNAR2
Input
IB: IFNAR2
IP: Biotin
IB: EGFR
Input
IB: EGFR
IP: Biotin
IB: α-Tubulin
Input
IB: α-Tubulin
e.
Isotype Control
1.1%
Control
43.8%
54
0.04%
IFNAR1
Control
ORF54
IB:FLAG
IB: α-Tubulin
1.0 ug/mL Tetracyline
+

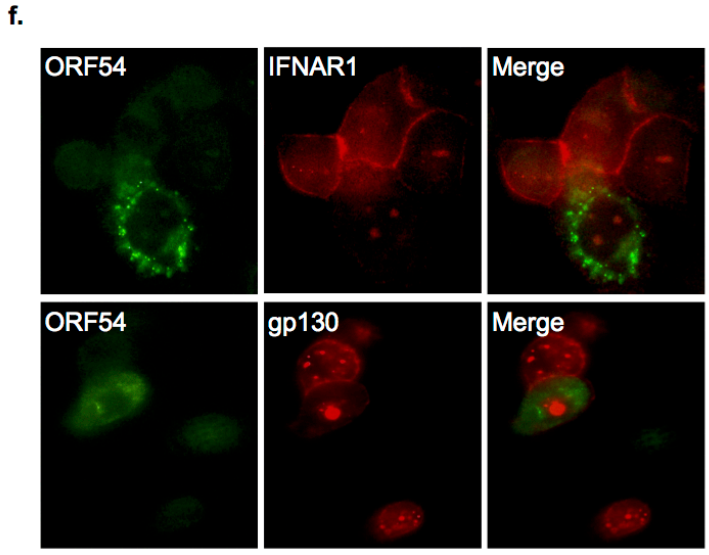
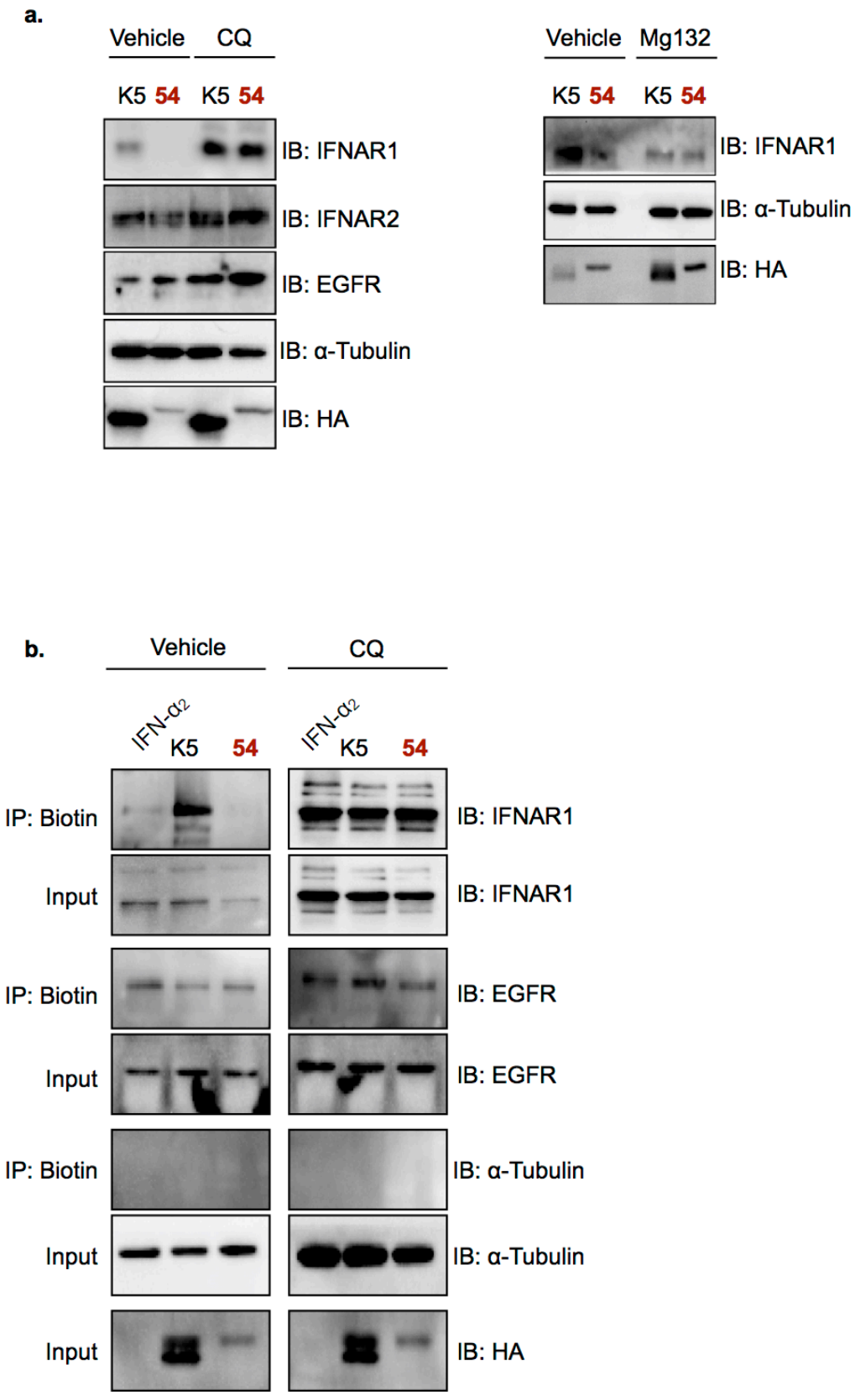
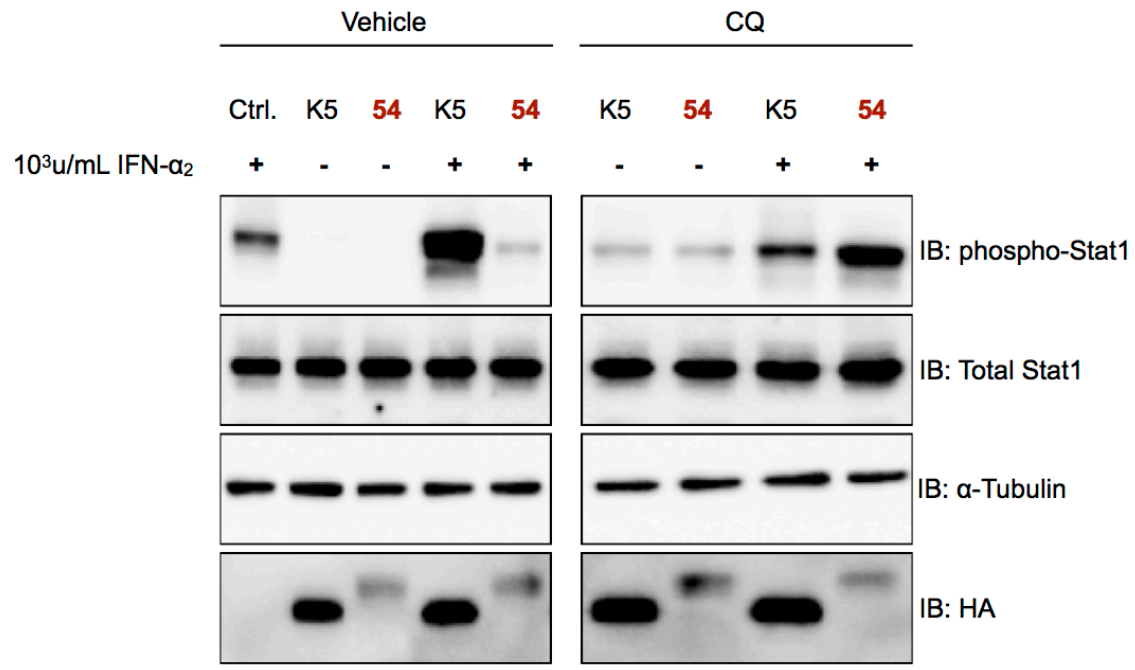
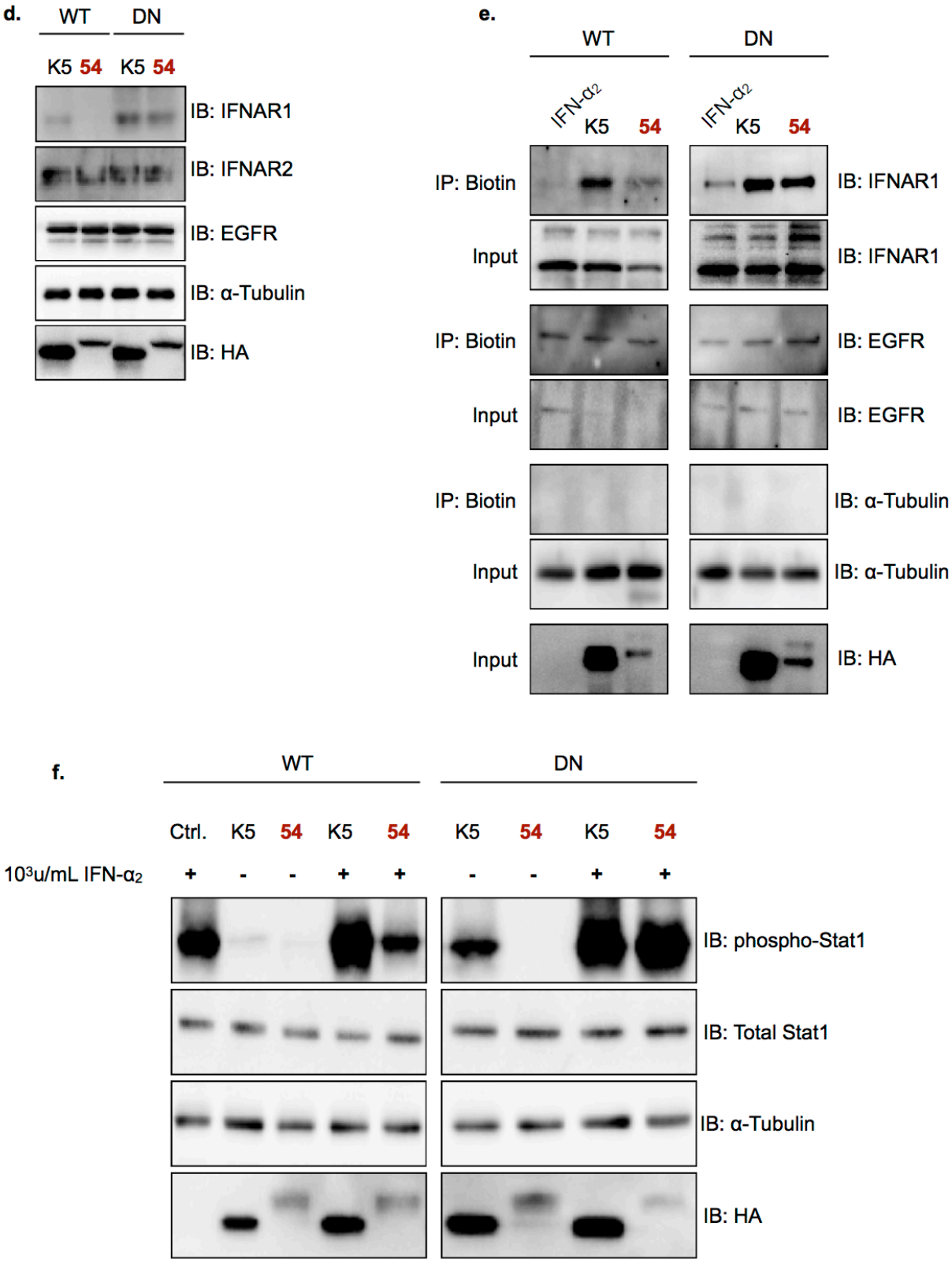

f.
ORF54
IFNAR1
Merge
ORF54
gp130
Merge

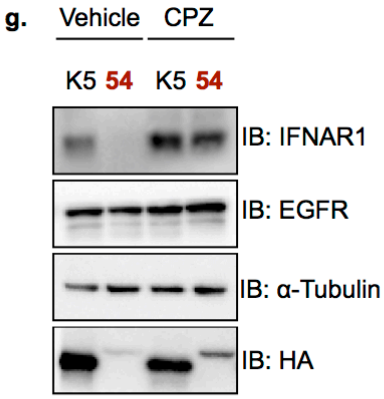
Figure 1. *ORF54 selectively targets IFNAR1 for degradation and reduction from the surface of the cell.* 293T and differentiated U937 cells were transfected with K5 as a negative control, ORF10 as a positive control (U937 only), and ORF54. ORF54 positive 293T and differentiated U937 cells show IFNAR1 degradation but not the membrane receptor EGFR (a). The degradation of IFNAR1 leads to the functional inhibition of the type-I IFN response as observed here by the significant reduction of ISG54 induction in the presence of ORF54 (b). Previous work determined that ORF54 does not transcriptionally regulate IFNAR1 REF. We measured the half-life of the protein in the presence of ORF54 and K5. Cells were treated with 20μg/mL of cycloheximide to inhibit ribosomal function and the translation of mRNA. This approach allowed us to evaluate the continued degradation IFNAR1 in the presence of ORF54, reducing the half-life of the receptor subunit by half. This suggests post-translational regulation of IFNAR1 by ORF54 (c). ORF54 induces the down-regulation of IFNAR1 and gp130 from the cell membrane. In (d) cells were transfected with ORF54 and K5 followed by surface biotinylation and precipitation of biotin via streptavidin. Via immunoblotting we observe a selective degradation of IFNAR1 and gp130, while IFNAR2 and EGFR remain stably expressed on the surface. Employing ORF54 inducible HeLa cells we are able to observe the reduction of e IFNAR1 from the surface of the cell membrane via Flow cytometry (e). Receptor down-regulation can also be observed in differentiated THP-1 cells using immunofluorescence microscopy (f). The data from these cell lines suggest that the degradation of IFNAR1 and gp130 leads to the reduction of receptor expression on the cell membrane, the functional site of these transmembrane receptors.

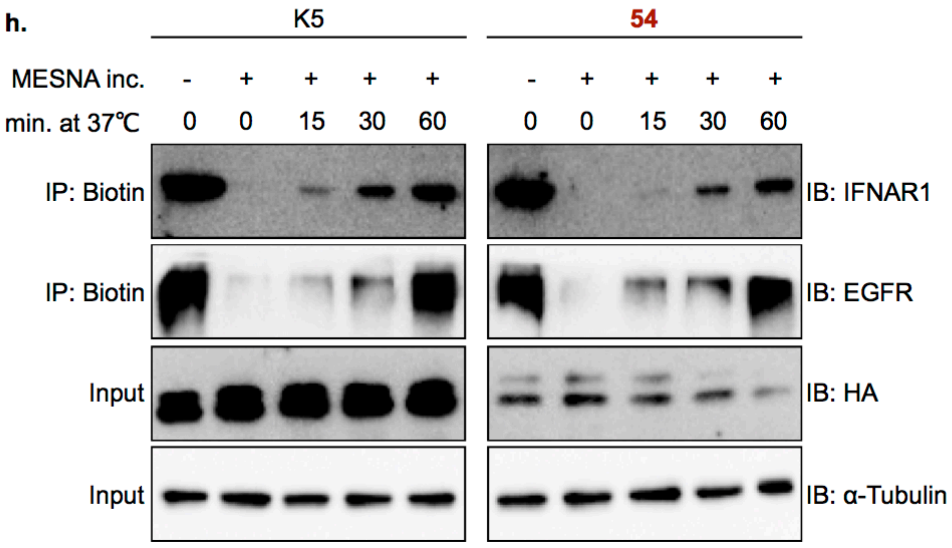

a.
Vehicle
CQ
K5 54 K5 54
IB: IFNAR1
IB: IFNAR2
IB: EGFR
IB: α-Tubulin
IB: HA
Vehicle
Mg132
K5 54 K5 54
IB: IFNAR1
IB: α-Tubulin
IB: HA
b.
Vehicle
CQ
IFN-α2
K5 54
IFN-α2
K5 54
IP: Biotin
IB: IFNAR1
Input
IB: IFNAR1
IP: Biotin
IB: EGFR
Input
IB: EGFR
IP: Biotin
IB: α-Tubulin
Input
IB: α-Tubulin
Input
IB: HA

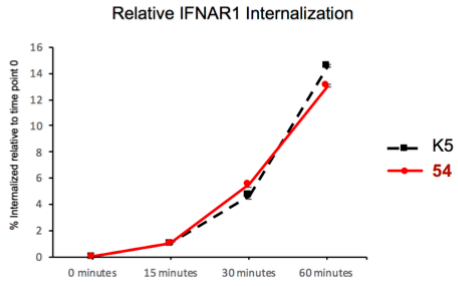
c.


Vehicle
CQ
Ctrl.
K5
54
K5
54
K5
54
K5
54
10³u/mL IFN-α₂
+
-
-
+
+
-
-
+
+
IB: phospho-Stat1
IB: Total Stat1
IB: α-Tubulin
IB: HA


d.
WT
DN
K5 54 K5 54
IB: IFNAR1
IB: IFNAR2
IB: EGFR
IB: α-Tubulin
IB: HA
e.
WT
DN
IFN-α2
K5 54
IP: Biotin
Input
IB: IFNAR1
IB: EGFR
IB: α-Tubulin
IB: HA
f.
WT
DN
Ctrl. K5 54 K5 54 K5 54 K5 54
10³u/mL IFN-α2
+ - - + + - - + +
IB: phospho-Stat1
IB: Total Stat1
IB: α-Tubulin
IB: HA


g.
Vehicle
CPZ
K5 54 K5 54
IB: IFNAR1
IB: EGFR
IB: α-Tubulin
IB: HA


h.
K5
54
MESNA inc. - + + + + - + + + +
min. at 37℃ 0 0 15 30 60 0 0 15 30 60
IP: Biotin
IP: Biotin
Input
Input
IB: IFNAR1
IB: EGFR
IB: HA
IB: α-Tubulin


Relative IFNAR1 Internalization
% Internalized relative to time point 0
0 2 4 6 8 10 12 14 16
0 minutes
15 minutes
30 minutes
60 minutes
K5
54

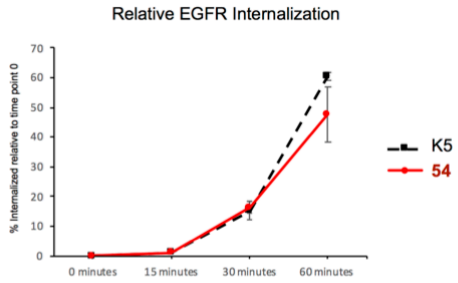
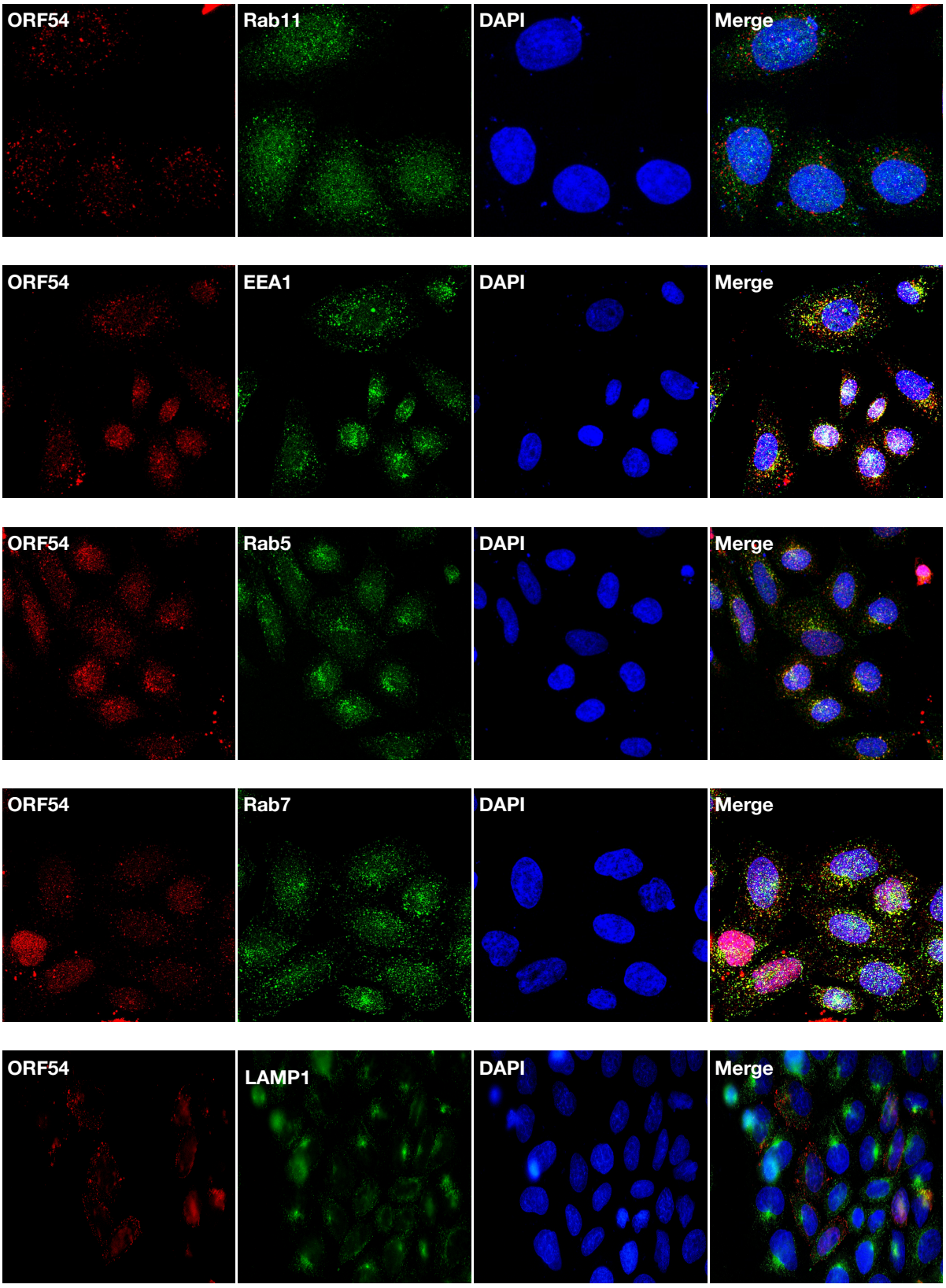

Relative EGFR Internalization
% Internalized relative to time point 0
0 10 20 30 40 50 60 70
0 minutes
15 minutes
30 minutes
60 minutes
K5
54

Figure 2. *ORF54 employs the ESCRT complex to shuttle IFNAR1 towards lysosomal degradation.* Transfecting cells with ORF54 results in the degradation of IFNAR1. ORF54 positive cells were treated with 50 μM of CQ for 12 hrs, which results in the rescue of IFNAR1 in the presence of ORF54. Of note is the associated upregulation of EGFR in the presence of CQ, indicating that inhibition of the lysosome provides general protection against degradation (a). We also noted that inhibition of the lysosome resulted in the general upregulation and rescue of IFNAR1 on the plasma membrane (b). This observation is confirmed by the successful reconstitution of the type-I IFN response measured via the induction of phosphorylated STAT1 (c). The experiments were repeated using the VPS4-DN that selectively inhibits decoupling of the ESCRT machinery. This genetic approach also resulted in the rescue of total IFNAR1 without the non-specific upregulation induced by the treatment CQ (d). Similarly, the inhibition of the ESCRT complexes results in the rescue of membrane bound IFNAR1 (e). Of note here is the rescue of IFNAR1 in the presence of ORF54, but only a partial rescue in the presence of IFN alpha2. This observation suggests that ligand induced biochemical changes induced on IFNAR1 result in a strong endocytosis signal; one, notably lacking in the presence of ORF54. We also observe the reconstitution of the type-I IFN response (f). While inhibition of the endocytosis via treatment with chlorpromazine (25 μM) predictably resulted in rescue of total IFNAR1 (g), a more significant observation is the lack of enhanced IFNAR1 uptake in the presence of ORF54 (h). This suggests to us that ORF54 does not induce the internalization of IFNAR1. Finally, we do not observe the enhanced phosphorylation of IFNAR1 in the presence of ORF54, while we do observe a loss of associated Tyk2 (i). This suggests that ORF54 does not induce the necessary biochemical changes to enhance IFNAR1's internalization, instead appears to take advantage of a basally endocytosed IFNAR1 that is less associated with Tyk2.


ORF54
Rab11
DAPI
Merge
ORF54
EEA1
DAPI
Merge
ORF54
Rab5
DAPI
Merge
ORF54
Rab7
DAPI
Merge
ORF54
LAMP1
DAPI
Merge

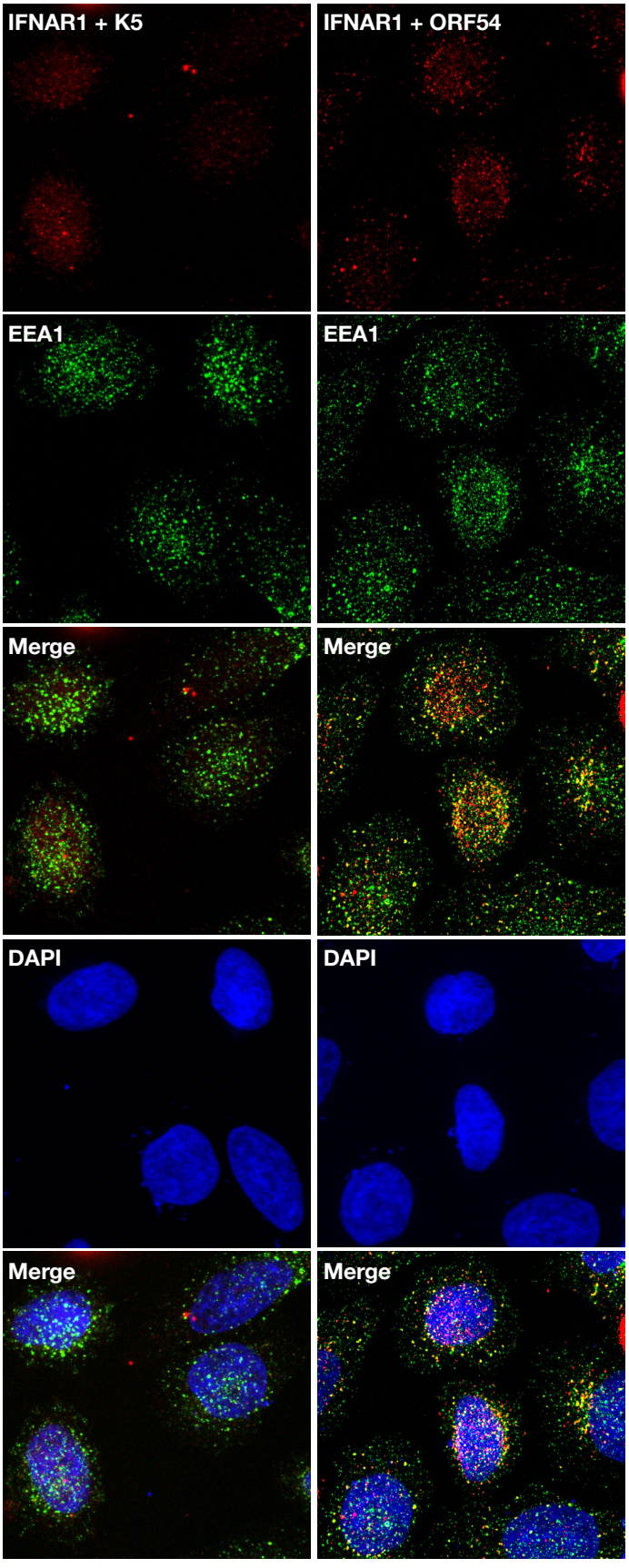
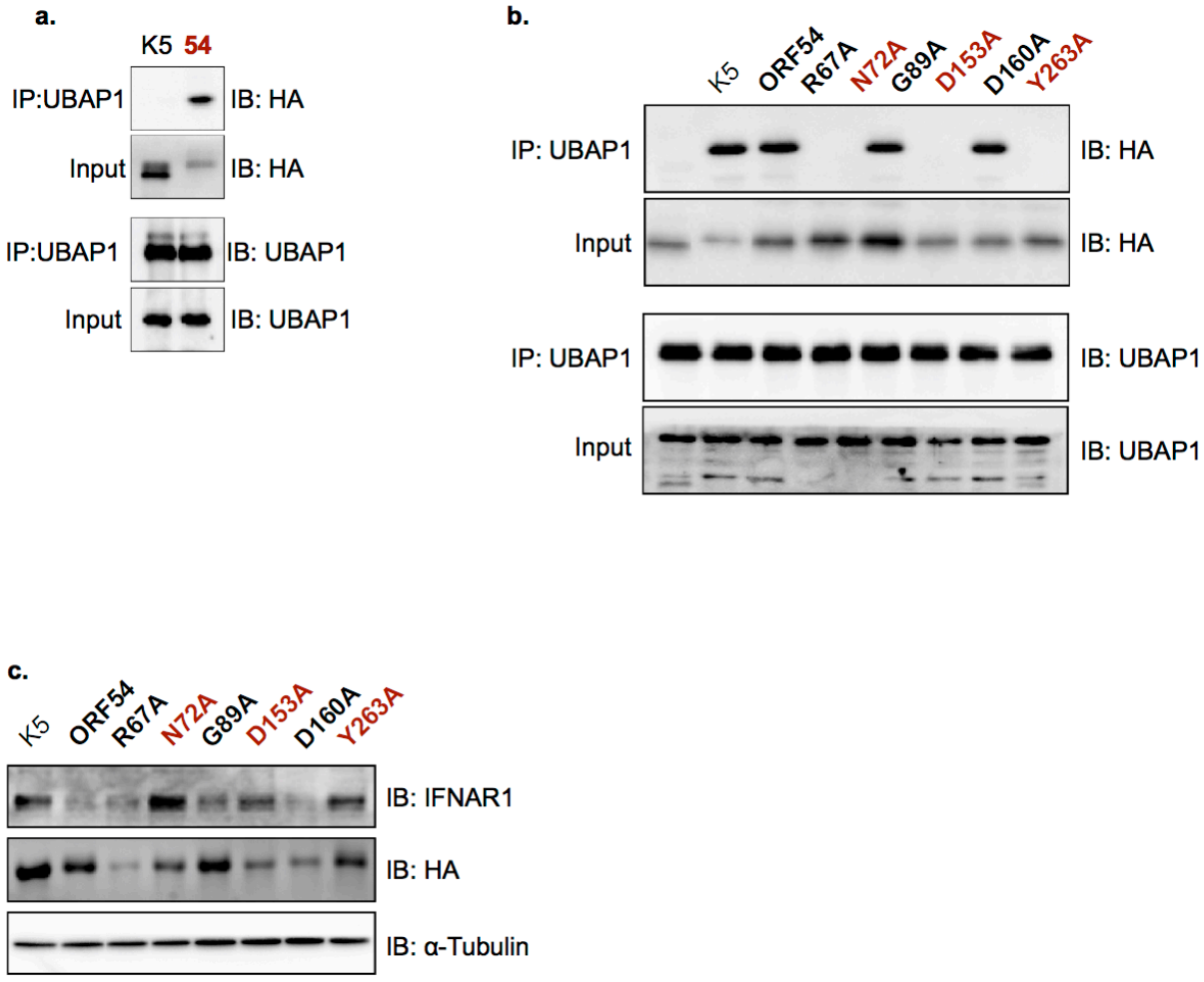
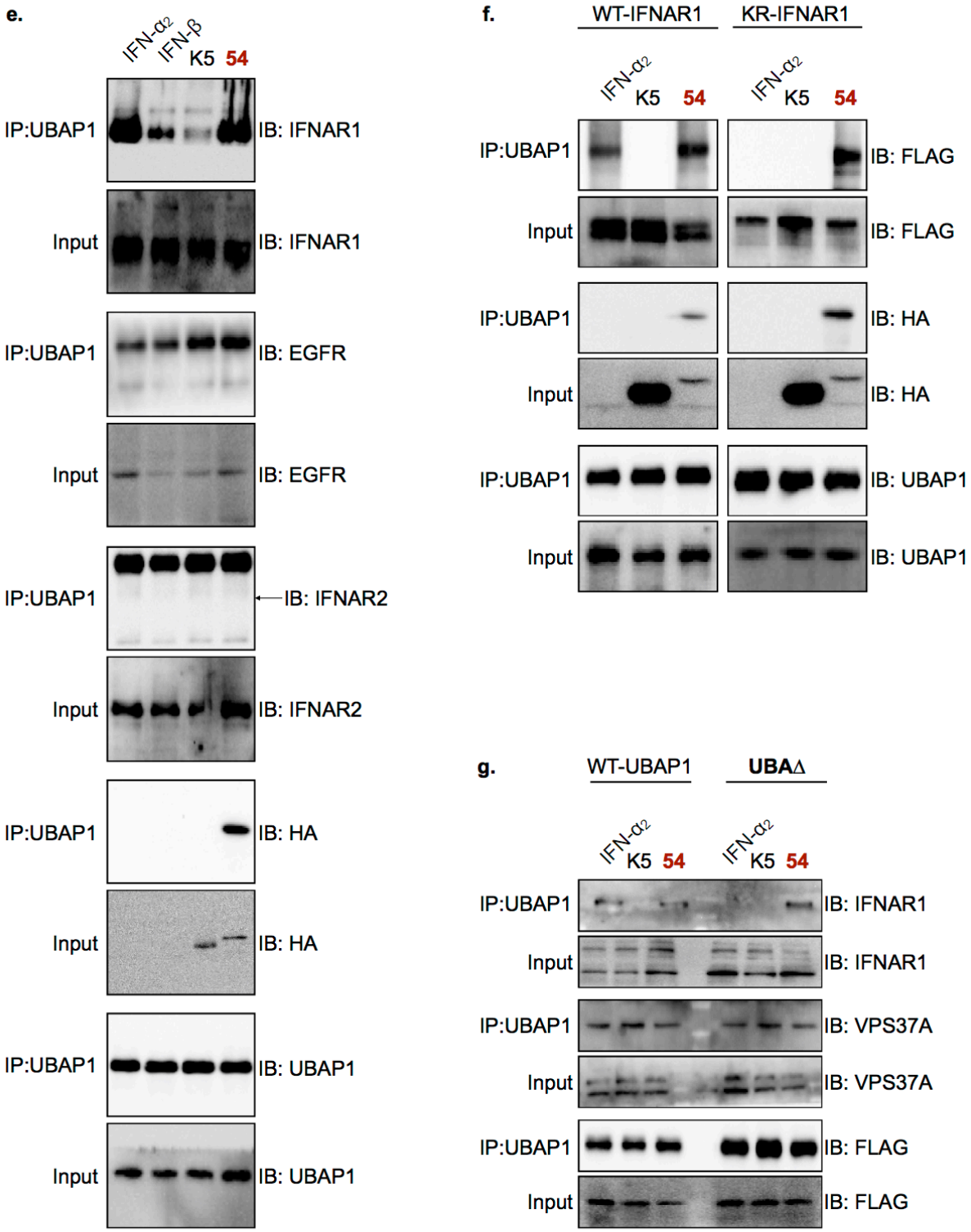

IFNAR1 + K5
IFNAR1 + ORF54
EEA1
EEA1
Merge
Merge
DAPI
DAPI
Merge
Merge

Figure 3. *ORF54 the IFNAR1's accumulation in the endosomal vesicle compartments.* HeLa cells were transfected ORF54, fixed and overnight stained for various vesicle markers. We determined that ORF54 overlaps with the early endosomal markers EEA1 and Rab5, as well as the late endosomal marker Rab7. This observation supports ORF54's location in the relevant endosomal compartments to influence receptor trafficking (panel a). Panel b displays the result of HeLa cells transfected with ORF54 or K5. We determined that in HeLa cells treated with ORF54 we observe an enhanced accumulation of IFNAR1 in vesicles also populated by ORF54. We concluded from these results that ORF54 mediates IFNAR1's accumulation in the endosome.


a.
K5
54
IP:UBAP1
IB: HA
Input
IB: HA
IP:UBAP1
IB: UBAP1
Input
IB: UBAP1
b.
K5
ORF54
R67A
N72A
G89A
D153A
D160A
Y263A
IP: UBAP1
IB: HA
Input
IB: HA
IP: UBAP1
IB: UBAP1
Input
IB: UBAP1
c.
K5
ORF54
R67A
N72A
G89A
D153A
D160A
Y263A
IB: IFNAR1
IB: HA
IB: α-Tubulin


e.
IFN-α2
IFN-β
K5
54
IP:UBAP1
IB: IFNAR1
Input
IB: IFNAR1
IP:UBAP1
IB: EGFR
Input
IB: EGFR
IP:UBAP1
IB: IFNAR2
Input
IB: IFNAR2
IP:UBAP1
IB: HA
Input
IB: HA
IP:UBAP1
IB: UBAP1
Input
IB: UBAP1
f.
WT-IFNAR1
KR-IFNAR1
IFN-α2
K5
54
IP:UBAP1
IB: FLAG
Input
IB: FLAG
IP:UBAP1
IB: HA
Input
IB: HA
IP:UBAP1
IB: UBAP1
Input
IB: UBAP1
g.
WT-UBAP1
UBAΔ
IFN-α2
K5
54
IP:UBAP1
IB: IFNAR1
Input
IB: IFNAR1
IP:UBAP1
IB: VPS37A
Input
IB: VPS37A
IP:UBAP1
IB: FLAG
Input
IB: FLAG

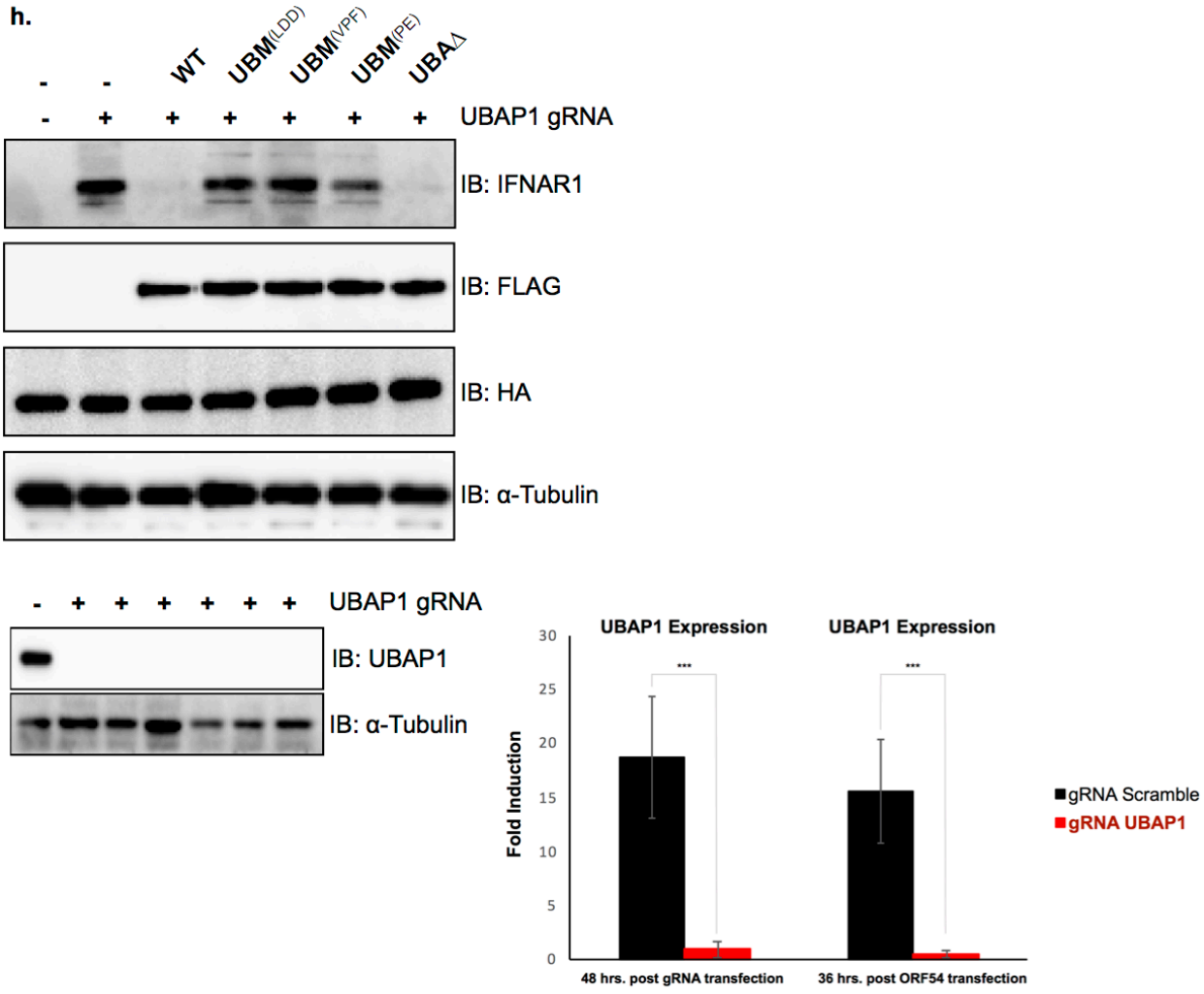
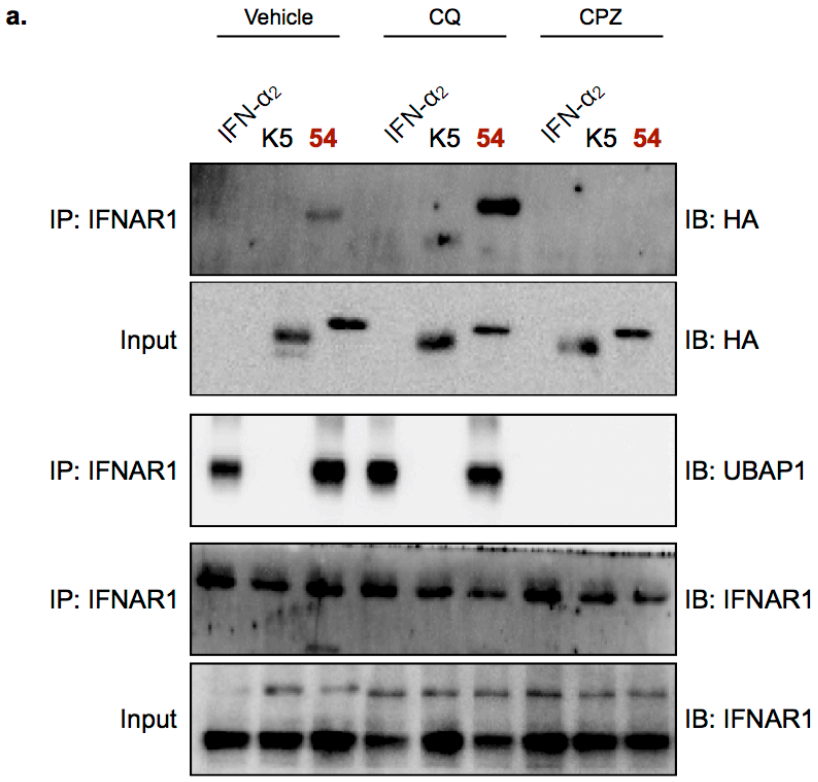
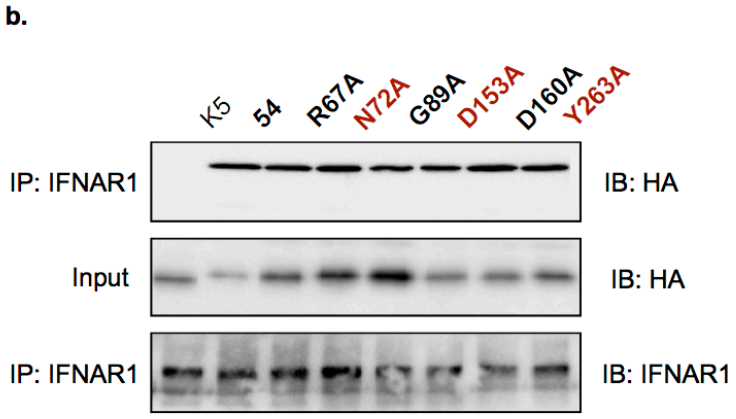
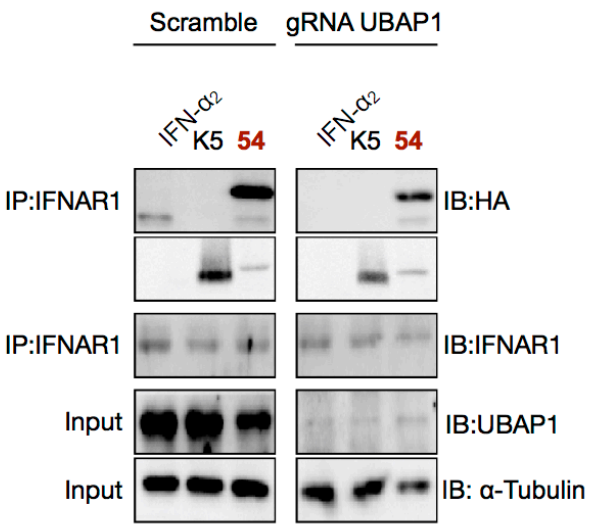

h.
WT
UBM(LDD)
UBM(IVPF)
UBM(PE)
UBAΔ
UBAP1 gRNA
IB: IFNAR1
IB: FLAG
IB: HA
IB: α-Tubulin
UBAP1 gRNA
IB: UBAP1
IB: α-Tubulin
UBAP1 Expression
UBAP1 Expression
Fold Induction
48 hrs. post gRNA transfection
36 hrs. post ORF54 transfection
gRNA Scramble
gRNA UBAP1

Figure 4. *UBAP1 is an essential host factor for ORF54-mediated down-regulation of IFNAR1.* We precipitated endogenous UBAP1 in cells transfected with ORF54 and K5. Probing for HA, we determined that UBAP1 associated with HA-tagged ORF54 (a). To evaluate UBAP1's functional relevance we performed association studies with ORF54 and ORF54-loss of function mutants that were identified via sequence alignment and mutation of conserved amino acid side chains (b,c). By employing these mutants, we determined that the loss of IFNAR1 down-regulation is observed corresponds to a loss of association with UBAP1 (b,c). This orthogonal approach establishes UBAP1's essential role to ORF54. We also evaluated the association between UBAP1 and IFNAR1 in the presence of ORF54. To that end, cells were transfected with ORF54 and K5, as well as treated with type-I IFNs as positive control. To measure the endogenous association between these proteins', cells were treated for 5 hrs with 25mM of CQ prior to IP. We determined that ORF54 as well as the type-I IFNs enhance the association between UBAP1 and IFNAR1 (e). This suggests that UBAP1's function is to tether the receptor subunit to the ESCRT complex. Of note is the observation that ORF54 enhances the association between IFNAR1 and UBAP1 independent IFNAR1 ubiquitination status. This observation is supported by Co-IP experiments maintaining the association between the KR IFNAR1 mutant and endogenous UBAP1 (f). Vice versa, the UBAP1 ubiquitin binding mutant maintains is association with endogenous IFNAR1 (g). The essential ability of UBAP1 to associate with ESCRT-I is further outlined by supplement experiments that replace endogenously KD UBAP1 with ESCRT- and ubiquitin binding mutants of UBAP1. We KD UBAP1 293T cells via the dCas9 system and then transfected cells with ORF54 and recombinant UBAP1 clones. IFNAR1 is continued to be degraded in cells expressing WT and UBAP1 ubiquitin binding mutant, but is rescued in cells co-expressing ORF54 and the UBAP1 ESCRT-I binding mutants. This observation exemplifies the significance of ESCRT binding to ORF54's mechanism (h).


a.
Vehicle
CQ
CPZ
IFN-α2
K5
54
IP: IFNAR1
IB: HA
Input
IB: HA
IP: IFNAR1
IB: UBAP1
IP: IFNAR1
IB: IFNAR1
Input
IB: IFNAR1


b.
K5
54
R67A
N72A
G89A
D153A
D160A
Y263A
IP: IFNAR1
IB: HA
Input
IB: HA
IP: IFNAR1
IB: IFNAR1

c.


Scramble
gRNA UBAP1
IFN-α2
K5
54
IFN-α2
K5
54
IP:IFNAR1
IB:HA
IP:IFNAR1
IB:IFNAR1
Input
IB:UBAP1
Input
IB: α-Tubulin

d.

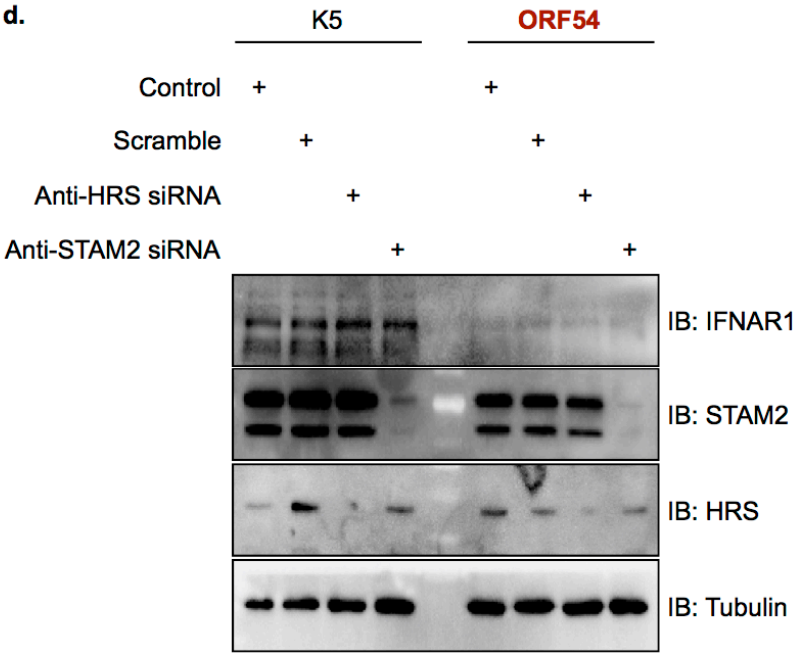

K5
ORF54
Control
Scramble
Anti-HRS siRNA
Anti-STAM2 siRNA
IB: IFNAR1
IB: STAM2
IB: HRS
IB: Tubulin

Figure 5. *ORF54 directs the endocytosed IFNAR1 to lysosomal degradation.* To evaluate the association between IFNAR1 and the viral dUTPase we transfected cells with ORF54 and K5. We determined that ORF54 associates with IFNAR1. This association is maintained in the presence of 50mM of CQ but interrupted by the inhibition of endocytosis via CPZ (a). Furthermore, ORF54 and treatment with IFN-2, facilitates the association with UBAP1. Of note is the interruption of the IFNAR1-UBAP1 association when endocytosis is inhibited (a). This observation facilitates the conclusion that ORF54 mediates the entry of IFNAR1 into the lysosomal degradation pathway, and that preventing endocytosis inhibits this function. We also aimed to characterize the association between ORF54 loss of function mutants and IFNAR1. We determined that all of ORF54's mutants maintain their association with IFNAR1 (b), thus suggesting that the receptor and viral protein interact with each other independently of UBAP1. We aimed to investigate the role of ESCRT-0 to the function of ORF54. This observation is further supported by the maintained association between ORF54 and IFNAR1 in the absence of via UBAP1 KD (c). We determined that KD of HRS and STAM2 does not rescue IFNAR1 in the presence of ORF54 (d), suggesting that the viral protein circumvents the ESCRT-0 complex to degrade IFNAR1.

Chapter Two: Evaluating ORF54's significance in the context of infection.

Summary

Research approach and emphasis

The focus of this chapter is a detail analysis and interpretation of data collected from experiments designed to evaluate the role of ORF54 during KSHV replication, as well as the evolutionary conservation of IFNAR1 down-regulation among herpesviruses. The majority of data presented herein is collected via KSHV-stably infected epithelial iSLK cells that allows for the reactivation of KSHV from latency. Reactivation of these cells leads to the lytic replication of the virus, allowing us to evaluate the role of ORF54 in KSHV lytic cycle. We constructed a KSHV mutant virus, 54S, unable to express the ORF54 protein via introducing a stop codon mutation. As negative controls we employed un-infected epithelial SLK cells and iSLK-puro cells that can be induced by doxycycline to express a viral transcriptional factor, RTA. The study on the ORF54 homologues encoded by other herpesviruses were performed with transfection of 293T cells. We included a KSHV ORF54 mutant that fails to interact with UBAP1, therefore unable to down-regulate IFNAR1 as a negative control.

Overview of observation

We observed that ORF54 facilitates the down-regulation of IFNAR1 during the lytic phase of KSHV. Furthermore, the degradation of IFNAR1 induced by ORF54 leads to a functional suppression of ISG54 expression, presumably limiting the antiviral activity of the type-I IFN response. We also aimed to validate the functional significance of UBAP1 to virally induced degradation of IFNAR1. We observed an increases in the IFNAR1 protein and IFN-mediated ISG54 induction in cells devoid of UBAP1 in the context of KSHV lytic replication. The reduction of viral lytic gene K8.1 was also noted in UBAP1-deficient lytic replication, which

was no observed for the lytic replication of 54S. This result indicates that UABP1 has an additional, previously uncharacterized role in KSHV lytic gene expression. Importantly, the lytic replication of 54S virus is more sensitive to the inhibition exerted by IFNα treatment, supporting the conclusion that ORF54 is essential for viral evasion of the type-I IFN response in order to replicate efficiently. Finally, we presented evidence to suggest that the ORF54 homologues of all herpesviruses possess the ability to down-regulate IFNAR1 via a conserved association with UBAP1.

Introduction

Chapter One outlines the detailed molecular study of ORF54 mediated antagonism of the type-I IFN response. Lacking is a clear understanding of ORF54's significance during viral infection. Herein, we evaluate the hypothesis that KSHV's ORF54 mediates degradation of IFNAR1 during lytic replication of the virus.

Overexpression of ORF54 provided essential details of the biochemical events and molecular mechanisms governing the selective degradation of IFNAR1. Our lab constructed a KSHV mutant unable to express ORF54 via a stop codon mutation to evaluate the function of ORF54. The goal of this approach is to evaluate the role of ORF54 in viral evasion of the type-I IFN response and viral replication. The antiviral activity of the type-I IFN response induces a robust antiviral state in the cell, limiting viral replication and spread. Previous work employing MHV68 cemented ORF54 significance to viral manipulation of the type-I IFN response and establishment of viral latency. Therefore, we propose that ORF54 plays a significant role in protecting KSHV from IFN during lytic replication.

It is of note that KSHV encodes multiple type-I IFN antagonists, and maintains a repertoire of other immune modulatory proteins. Thus, the elimination of ORF54 may not result in a

significant loss to KSHV replication. ORF54 is expressed as an early gene during KSHV lytic replication [2]. However, unlike most of other herpesviruses, *de novo* infection of KSHV leads to latent infection for most of cases. Lytic replication of KSHV is usually studied by reactivating KSHV in latently-infected cell lines using chemicals such as sodium butyrate (NaB), a known inhibitor of histone deacetylase (HDAC) activity. The bacterial artificial chromosome (BAC) system has allowed us to efficiently manipulate the large viral DNA genome. Recently, we constructed the KSHV mutant with the translation stop codon at amino acid 30 of ORF54 based on a KSHV BAC plasmid, rKSHVBAC16 (a gift from Dr. Jae Jung's group). The rKSHVBAC16 plasmid is derived from rKSHV.219[101] and has the insertion of a cassette controlled by the cellular EF-1alpha promoter to express GFP and the hygromycin resistant gene product from IRES. The 54S KSHV BAC plasmid was transfected into 293T cells to reconstitute the 54S virus. We employed an epithelial cell line (iSLK) that was established to express inducible RTA [1], a viral transcriptional activator that is necessary and sufficient to induce KSHV lytic replication for stable latent infection of KSHV. To reactivate the virus, cells can be either treated with doxycycline alone to induce RTA or in combination with NaB. The combination results in a uniform and complete activation of KSHV lytic replication. Treatment of doxycycline alone leads to a lower level of KSHV reactivation with a slower kinetics than the combination. Similar to MHV-68, ORF54 is not required for KSHV lytic replication. We were able to generate 54S-latently infected iSLK cell line (iSLK-54S). Our goal here is to evaluate whether during KSHV lytic replication IFNAR1 expression and the responses to exogenous IFNα are reduced by ORF54.

Results

ORF54 mediates IFNAR1 down-regulation during KSHV infection

Herpesviral genes are expressed in a cascade manner, immediate early, early, and late. At least one additional gene, ORF10, was found to down-regulate IFNAR1 (Fig. 1-1). Therefore, we harvested cells at various time points post-induction with dox and NaB (activated). The latently infected cells without activation were included as a control (Figure 2-1a). We showed that increased IFNAR1 expression in the activated iSLK-54S cells compared to iSLK-WT at 24 hours post-activation. This difference was lost at later times, presumably due to the expression of other viral IFN antagonists, such as ORF10. Expression of K8.1, a viral late gene, was comparable between WT and 54S. Therefore, under induction of dox and NaB, the lack of ORF54 has minimal impact on KSHV lytic replication.

We also investigated the impact of IFNAR1's degradation on the induction of the type-I IFN response by examining the transcript of ISG54. It was previously shown that KSHV lytic replication induces little IFNβ[102]. Thus, to determine how infected cells respond to type I IFNs, we treated cells with IFNα and harvested total RNA for RT-PCR. Clearly, with KSHV replication, the cells became less responsive to IFNs, indicated by lower induction of ISG54. The induction of ISG54 is significantly higher in activated iSLK-54S cells (Figure 2-1b), but not to the level as unactivated cells, despite that IFNAR1 is fully rescued. This is likely due to other viral genes that can inhibit ISG induction. For example, RTA expression alone in activated iSLK-puro also moderately affected ISG54 induction. Nevertheless, our data support a functionally significant role of ORF54 play in the degradation of IFNAR1 and suppression of type-I IFN responses at early times during KSHV lytic replication.

UBAP1 is required for KSHV to down-regulate IFNAR1

Next, we investigated the function of UBAP1 in the context of infection. To that end, we knocked down UBAP1 using siRNA. Following UBAP1 suppression, we activated iSLK-WT to via doxy and NaB. Knockdown of UBAP1 rescues IFNAR1 expression (Figure 2-1c) , supporting its critical role in down-regulation of IFNAR1 during KSHV lytic replication. While ligand-induced EGFR degradation is also mediated by UBAP1, we did not observe increased EGFR expression by UBAP1 knockdown.

We noted that UBAP1 knockdown reduced K8.1 expression (Figure 2-1c). This observation suggests that UBAP1 is important for efficient viral lytic replication. It is tempting to ascribe this phenotype to more abundant IFNAR1 and thus a possibly more robust IFN response against KSHV, limiting its replication. However, we did not see such an effect in activated iSLK-54S cells despite of fully rescued IFNAR1 expression. UBAP1 is known to be required for K5-mediated down-regulation of tetherin, which may interfere with virion production. Our result indicated that UBAP1 may have an additional role in KSHV lytic replication.

Following this observation, we carried out a follow-up experiment evaluating the consequence of UBAP1 knockdown on IFN responses. Cells were treated with IFNα and the ISG54 transcript was measured. Similar to what we observed for activated iSLK-54S cells, there was more ISG54 expression in activated iSLK-WT cells when UBAP1 expression was knocked down (Figure 2-1d).

ORF54 is critical for KSHV to counteract type I IFN responses

Next, we determined whether viral replication of 54S became more susceptible to antiviral effects of IFN than WT. To that end, we examined K8.1 expression in activated cells treated with IFNα. Treatment at 24 hr post-induction reduced K8.1 expression in activated iSLK-54S

compared to iSLK-WT. However, this reduction was not observed when IFNα treatment started at 36 hr post-induction. Therefore, the significance of the ORF54's anti-IFN function is time-dependent. The evidence provided by these experiments supports the model in which at early times during KSHV lytic replication ORF54 is essential for viral evasion of the type-I IFN response by down-regulating IFNAR1 via UBAP1.

The anti-IFN function is evolutionarily conserved among herpesviral dUTPases

Mammalian herpesviruses are divided into three subfamiles, alpha, beta, and gamma. Homologues of ORF54, or dUTPases, are encoded by all three subfamilies. The alphaherpesvirus Pseudorabies Virus (PRV) homolog encoded by UL50 was recently shown to induce degradation of IFNAR1 as well[103]. Here we investigated whether other homologues also down-regulate IFNAR1. These include the EBV BLLF3 and UL72 of cytomegalovirus, a beta-herpesvirus. We showed that in addition to UL52, BLL3 and UL72, all three dUTPase homolgoues reduce the IFNAR1 protein to an almost undetectable level as ORF54 (Figure 2-2a). Included as a control is an ORF54 mutant (N72A) that fails to down-regulate IFNAR1 due to the loss of its interaction with UBAP1.

Furthermore, we determined whether these dUTPase proteins also associate with UBAP1. Co-IP experiments were performed by using anti-UBAP1 antibodies and probed for the viral dUTPase. As a control, we employed the N72A mutant. UBAP1 is shown to interact with all four herpesviral dUTPases tested. The data indicate that herpesviral dUTPase has evolved a novel, evolutionarily conserved mechanism to counteract IFN-I responses.

Discussion

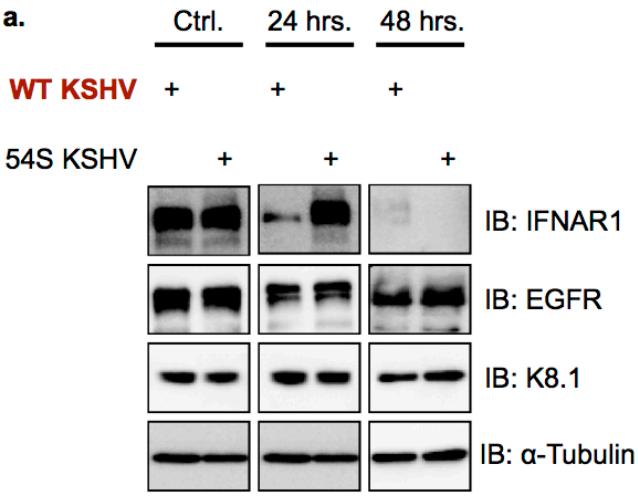
ORF54 functions to limit the type-I IFN response during infection.

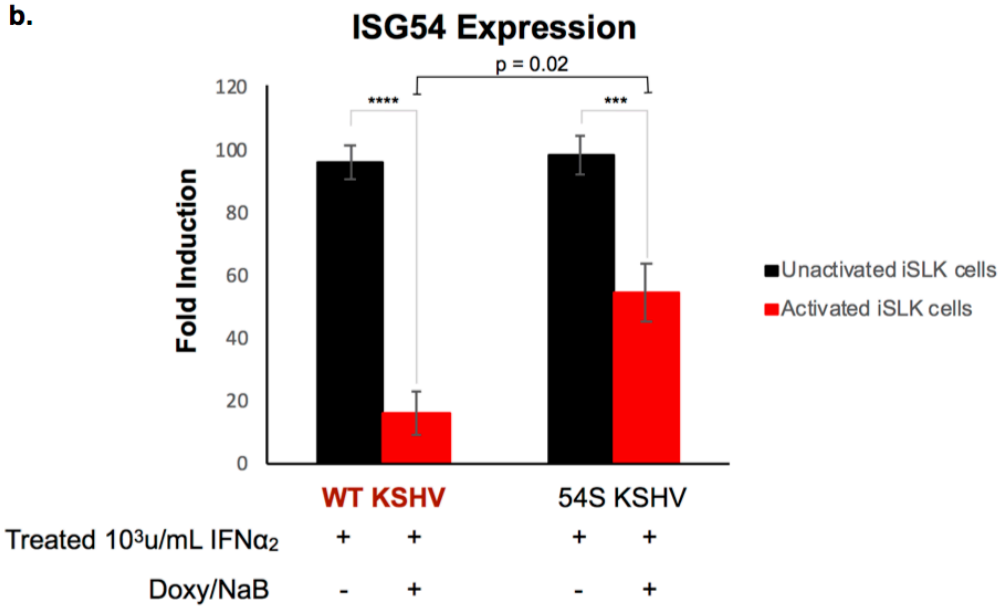
During KSHV reactivation ORF54 contributes to counteracting type-I IFN responses for effective viral lytic replication. It is noted that the difference between WT and 54S in their ability to down-regulate IFNAR1 and IFNα signaling is limited to a small window (Supplementary figure). At later time points post-induction, the IFNAR1 protein level is comparably reduced between WT and 54S and so is its IFNα-induced ISG54 expression. Accordingly, viral replication of 54S became resistant to IFNα as WT at later times after more viral genes are expressed. On the other hand, IFNα treatment initiated before viral reactivation is capable of inhibiting KSHV replication [102]. KSHV encodes multiple genes to escape IFN control like other herpesviruses. For example, ORF10, a delayed early gene, can inhibit IFN-I signaling [104]. Moreover, KSHV encodes a SOX protein that induces global RNA degradation[105-106], which can also impact the protein level of IFNAR1 whose half-life is only a few hours. Our study clearly demonstrates an early, time-dependent role of ORF54 in escaping IFN control. Because IFNAR1 constitutively undergoes internalization (Fig), ORF54 provides a mechanism to rapidly alter surface expression of IFNAR1 by sorting the internalized IFNAR1 to the MVB/lysosome pathway. KSHV reactivation induces very little IFN-I[102], thus, ORF54 likely protects the virus from IFN-Is produced by responding immune cells before a full bloom of viral gene expression. In the future, it will be important to determine whether the ORF54 homologues of other herpesviruses play a similar function in the context of viral replication.

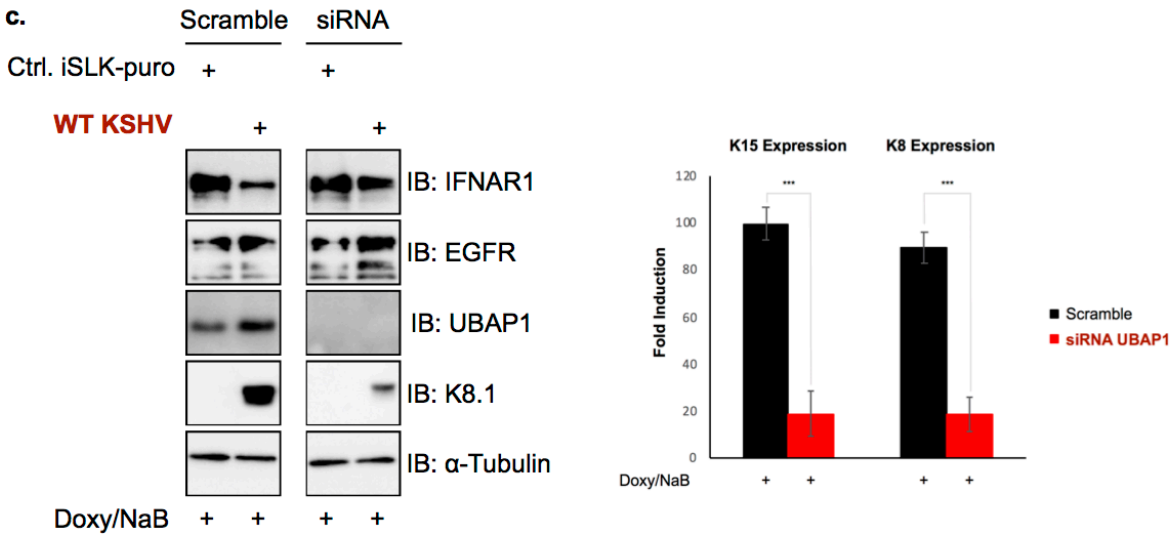
Targeting constitutively internalized IFNAR1 may be conserved among other herpesviruses

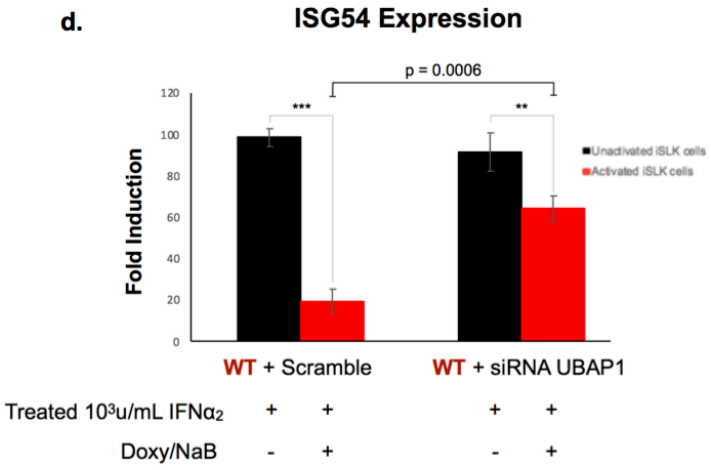
We observed that the mechanism of KSHV's ORF54 is conserved among herpesviruses. ORF54 and its homologs are structurally-related dUTPases, which likely have evolved from a captured

cellular counterpart. Previous work by us and others has shown that viral dUTPase while dispensable for replication *in vitro* provide advantages to viruses *in vivo*[68]. In addition, we further demonstrated in MHV-68 that the *in vivo* function of viral dUTPase is to negate type-I IFN responses and such a function does not depend on its enzymatic activity in nucleotide metabolism[68]. Subsequently, similar to what we observed in MHV-68, others also found that dUTPases encoded by KSHV[69] and alphaherpesviruses[103] affect IFNAR1 expression. Unlike the dUTPases encoded by alpha- and gammaherpesviruses, the CMV's UL72 is catalytically inactive, and its function for viral replication has remained ambiguous[107]. Our data provide evidence that UL72 function in the down-regulation of IFNAR1 and attenuation of the type-I interferon response. Taken together, our results suggest that herpesviral dUTPases have acquired a conserved mechanism of action that employs UBAP1 as a key host factor to facilitate the degradation of cytokine receptors, such as IFNAR1, independent of its nucleotide hydrolyzing activity.


a.
Ctrl.
24 hrs.
48 hrs.
WT KSHV + + +
54S KSHV + + +
IB: IFNAR1
IB: EGFR
IB: K8.1
IB: α-Tubulin


b.
ISG54 Expression
p = 0.02
****
***
Fold Induction
120
100
80
60
40
20
0
WT KSHV
54S KSHV
Unactivated iSLK cells
Activated iSLK cells
Treated 10³u/mL IFNα₂ + + + +
Doxy/NaB - + - +


c.
Scramble
siRNA
Ctrl. iSLK-puro + +
WT KSHV + +
IB: IFNAR1
IB: EGFR
IB: UBAP1
IB: K8.1
IB: α-Tubulin
Doxy/NaB + + + +
K15 Expression
K8 Expression
Fold Induction
120
100
80
60
40
20
0
***
***
Doxy/NaB + + + +
Scramble
siRNA UBAP1


d.
ISG54 Expression
p = 0.0006
Fold Induction
120
100
80
60
40
20
0
***
**
Unactivated iSLK cells
Activated iSLK cells
WT + Scramble
WT + siRNA UBAP1
Treated 10³u/mL IFNα₂ + + + +
Doxy/NaB - + - +

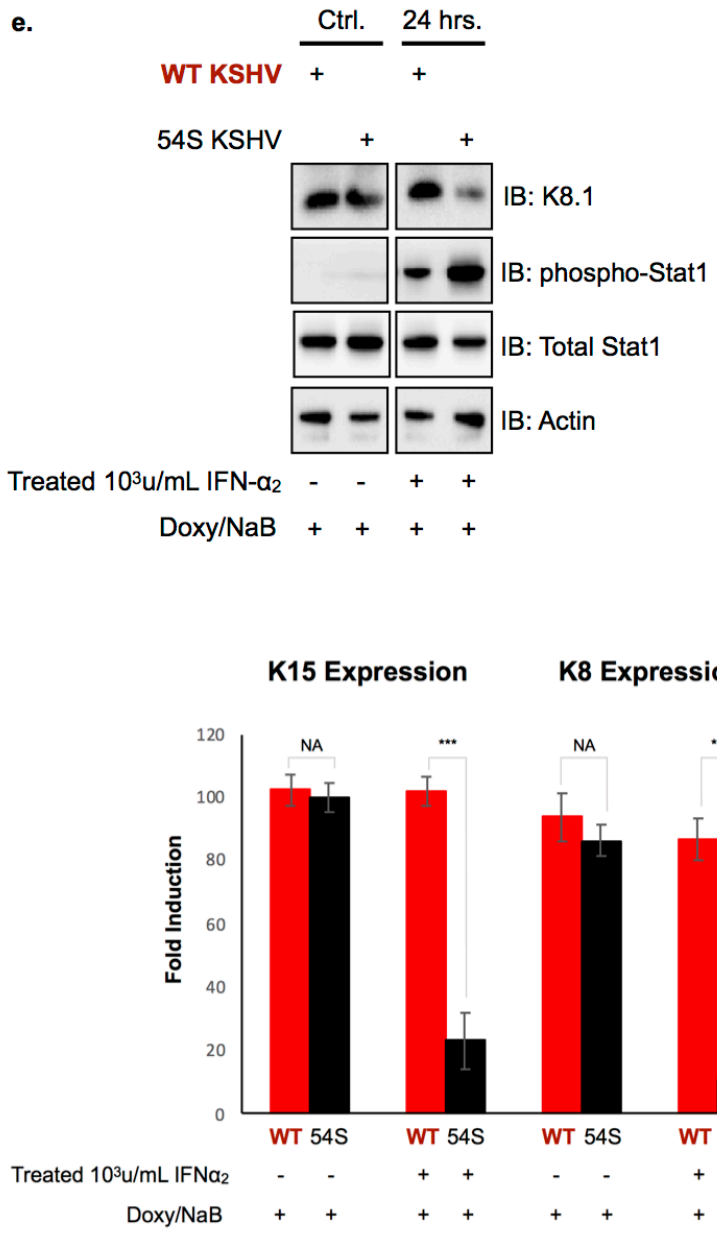

e.
Ctrl.
24 hrs.
WT KSHV + +
54S KSHV + +
IB: K8.1
IB: phospho-Stat1
IB: Total Stat1
IB: Actin
Treated 10³u/mL IFN-α₂ - - + +
Doxy/NaB + + + +
K15 Expression
K8 Expression
Fold Induction
120
100
80
60
40
20
0
NA
***
NA
***
WT 54S
WT 54S
WT 54S
WT 54S
Treated 10³u/mL IFNα₂ - - + + - - + +
Doxy/NaB + + + + + + + +
WT KSHV
54S KSHV

Figure 1. *ORF54 mediates IFNAR1 down-regulation of during KSHV infection.* To evaluate the expression of IFNAR1 in the presence of WT- and 54S-KSHV we induced iSLK cells via addition of 5μg/mL of doxycycline and 1mM Sodium butyrate. 24hrs post-induction, cells were harvested and lysates prepared for immunoblotting. As can be observed in (a) WT-KSHV efficiently down-regulates IFNAR1. In contrast, virus lacking ORF54 is unable to down-regulate IFNAR1 24hrs post-induction. Evaluating the strength of the antiviral response we treated cells with 1000U/mL of exogenous IFN-α and harvested total RNA at 24hrs post-induction. We measured the induction of ISG54 and determined that significantly less is expressed in the presence of WT virus compared to 54S virus (b). We also aimed to evaluate the role of UBAP1 in the context of infection. To that end, we KD UBAP1 prior to reactivation of virus. We observed the rescue of IFNAR1 in the absence of UBAP1 (c). We also observed a reduction of the lytic gene K8.1, suggesting less virus able to efficiently replicate. Evaluating the functional significance of IFNAR1's rescue we treated cells with 1000U/mL of exogenous IFN-α and harvested total RNA at 24hrs post-induction. We observed a significant upregulation of ISG54 when UBAP1 is KD (d), suggesting that the increased abundance of IFNAR1 is functionally capable of reconstituting the type-I IFN response. We hypothesized that virus lacking ORF54 is more sensitive to exogenously applied IFN-α. We observed that the 54S-KSVH expressed significantly less K8.1, suggesting viral sensitive to the antiviral response induced by IFN-α.

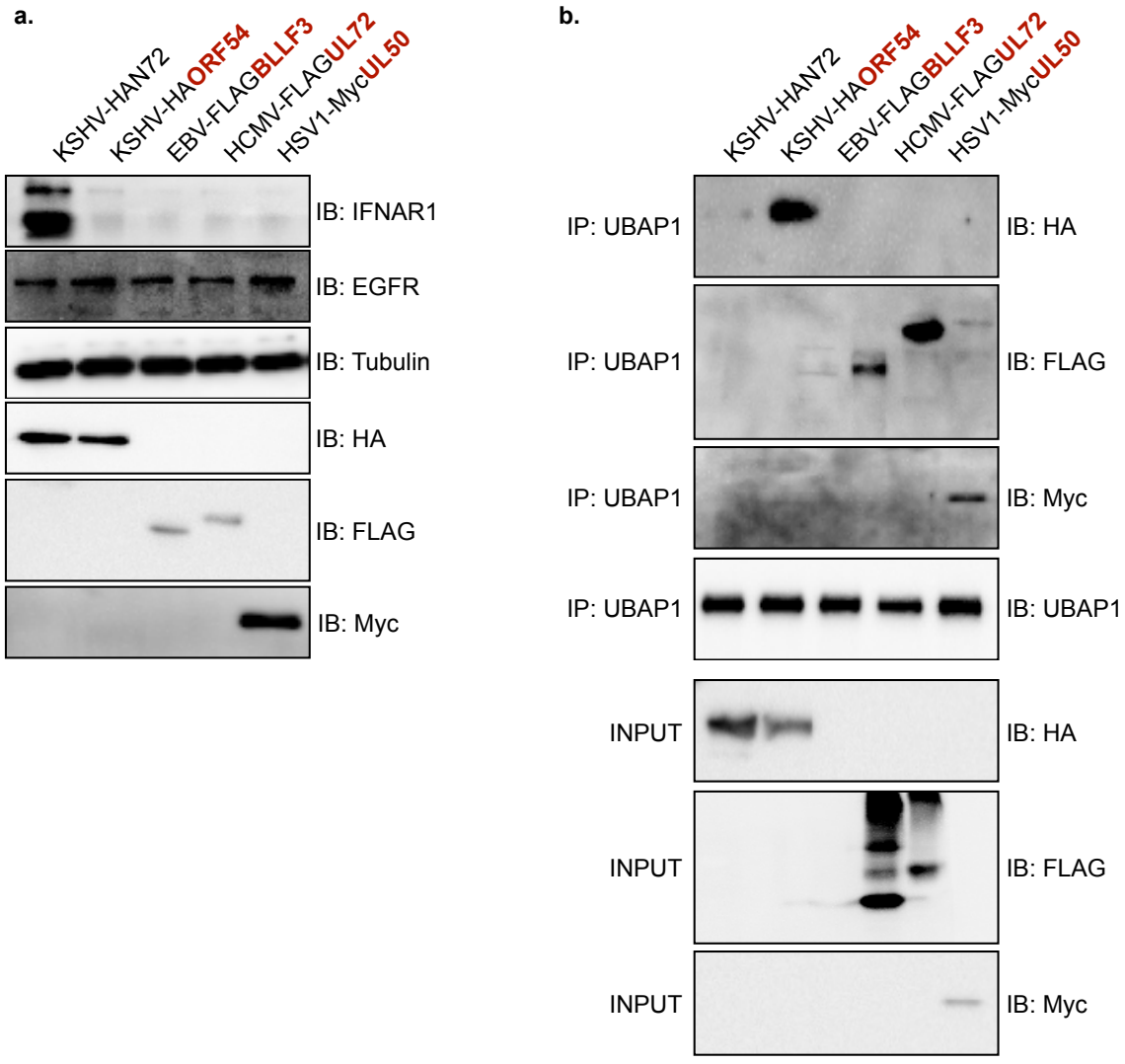

a.
KSHV-HAN72
KSHV-HAORF54
EBV-FLAGBLLF3
HCMV-FLAGUL72
HSV1-MycUL50
IB: IFNAR1
IB: EGFR
IB: Tubulin
IB: HA
IB: FLAG
IB: Myc
b.
KSHV-HAN72
KSHV-HAORF54
EBV-FLAGBLLF3
HCMV-FLAGUL72
HSV1-MycUL50
IP: UBAP1
IB: HA
IP: UBAP1
IB: FLAG
IP: UBAP1
IB: Myc
IP: UBAP1
IB: UBAP1
INPUT
IB: HA
INPUT
IB: FLAG
INPUT
IB: Myc

Figure 2. *An evolutionarily conserved function of Herpesvirus dUTPase proteins.* We evaluated the capability of HVs homologs of KSHV's ORF54 to down-regulated IFNAR1 in 293T. We overexpressed recombinant proteins in 293T cells and harvested cells 36hrs post-transfection and prepared lysates for WB. We determined that IFNAR1 is selectively degraded in the presence of the HV dUTPases, suggesting a conserved type-I IFN antagonism mechanism (a). We also determined that all HV homologs retain their ability to associate with UBAP1. Suggesting UBAP1 is a critical host factor for the manipulation of the host type-I IFN response by herpesviruses (b).

Chapter Three: ORF54 mediated IFNAR1 ubiquitination.

Summary

Research approach and emphasis

The focus of this chapter is a preliminary analysis and interpretation of experimental results aimed to evaluate the ubiquitination of IFNAR1 and functional relevance of HECTD1. Experiments focusing on the ubiquitination status of IFNAR1 employ a specific ubiquitination immunoprecipitation that eliminates non-covalent associations from the IP. This result is achieved via rapid lysis of cells, followed by boiling at 95°Cta. Of note, is the continued use of CQ to enrich for the ubiquitinated species of IFNAR1. In the absence of CQ treatment IFNAR1 is degraded following the addition of IFNα and in the presence of ORF54. Throughout the experimental procedures, K5 acts a negative control. As a viral E3 ligase involved in the ubiquitination of tethrin, resulting in the endosomal sorting via UBAP1, it is a critically relevant function negative control.

We also carried out a preliminary evaluation of the functional significance of HECTD1, an E3 ligase identified through the yeast two-hybrid screening. To examine the association between ORF54 and HECTD1, we employed a Bi-FC approach instead of a Co-IP before we can successfully clone the large size of HECTD1. Furthermore, the antibody against the endogenous E3 ligase is not suited for Co-IP experiments. As such, we resorted to sub-clone the potential interaction domain of HECT1. Critically, the E3 ligase domain acts as a negative control as it is unable to associate with anything except ubiquitin.

Introduction

We have previously observed the lysosomal degradation of IFNAR1 in the presence of ORF54. Work done by Kumar et al.[71] determined that the RING E3 ligase ubiquitinates IFNAR1 in the presence of the type-I IFNs. The ubiquitination at the cytoplasmic tail of IFNAR1 is a critical sorting signal that leads to its degradation in the lysosome[70].

Commonly, the ubiquitination chains resulting from repeated covalent attachments of ubiquitins via the amino acid side chains of lysines 63 (K63) and 48 (K48) found on the ubiquitin protein result in the critical recognition by ubiquitin-binding proteins [81-82,109]. Binding of these adaptor proteins results in the shuttling of ubiquitinated cargo towards proteasomal or lysosomal degradation. As such, ubiquitination plays an essential role in marking proteins for degradation[109].

We observed that ORF54 enhances IFNAR1's association with UBAP1 independent of ubiquitination. This observation suggests that ORF54 facilitates the receptor subunit entry into the ESCRT-mediated degradation pathway circumventing the need for ubiquitination. This allows ORF54 to bring un-ubiquitinated IFNAR1 not designated for degradation into the lysosomal pathway. Our research, however, indicates that the lysine mutant of IFNAR1 (KR-IFNAR1), while associated with UBAP1 in the presence of ORF54, remains resistant to ORF54-mediated degradation. We, therefore, concluded that ubiquitination of IFNAR1 is not required for sorting but still a requirement for its degradation.

Herein, we evaluate the question of IFNAR1's ubiquitination status in the presence of ORF54 via the immunoprecipitation of the receptor and ubiquitin. Furthermore, we employ a ubiquitin K63/48 mutant that is unable to form ubiquitination chains via these lysines. Although seven lysine residues on ubiquitin have been identified to signal protein translocation; canonically

recognized, the K63/K48 ubiquitin chains are known to induce proteasomal and lysosomal degradation of tagged proteins. As such we evaluate the ability of ORF54 to induce IFNAR1's ubiquitination in the presence of this ubiquitin mutant.

ORF54 does not maintain an active E3 ligase domain, as such, we evaluated known ORF54 interaction partners for potential E3 ligases that facilitate IFNAR1's ubiquitination. The previously mentioned Y2H screen determined that ORF54 interacts with HECTD1, a recently identified E3 ligase that facilitates the ubiquitination of heat shock protein 90 (Hsp90)[110-113]. We evaluated IFNAR1's degradation in the absence of HectD1 as a preliminary evaluation of HectD1 function in the ORF54 mechanism.

Results

ORF54-mediated degradation of IFNAR1 requires ubiquitination.

Ubiquitination is an essential sorting signal to the ESCRT-mediated lysosomal degradation. We determined that the association between IFNAR1 and UBAP1 in the presence of ORF54 is independent of ubiquitin by utilizing a lysine IFNAR1 KR mutant devoid of ubiquitination sites within the cytoplasmic tail. However, the KR mutant is resistant to ORF54-mediated degradation (Figure 3-1a). Therefore, ubiquitination is still required for successful degradation. Here we further characterize the ubiquitination of IFNAR1 in the presence of ORF54. We evaluated IFNAR1's ubiquitination status by overexpressing FLAG-tagged IFNAR1 and HA-tagged ubiquitin. As controls, cells were treated with IFNα, a known inducer of IFNAR1 ubiquitination, and K5, our negative control viral E3 ligase. When we immune-precipitated IFNAR1 (FLAG-IFNAR1) with the anti-FLAG antibody and probed for ubiquitin by the anti-HA antibody we showed that there was more ubiquitinated IFNAR1 in the presence of IFNα or ORF54 compared to K5 (Figure 3-1b).

Next, we evaluated the ubiquitination of IFNAR1 using the K63/48 mutant of ubiquitin. Similarly, we over-expressed the FLAG-tagged IFNAR1 and HA-tagged ubiquitin mutant. The expression of the ubiquitin mutant stunts the ubiquitin chain extension, limiting the ubiquitination of targeted proteins for both IFNα treatment and ORF54 expression (Figure 3-1d). This indicates that ubiquitination of IFANR1 at these two conditions is primarily through extension at lysine K63 or K48.

The KR mutant has lysines altered at 501, 525 and 527 that were previously determined to be important for IFN-induced ubiquitination. Also included in this study is the SR mutant that has serines altered at 535 and 539. These two serine residues are essential for signaling events that

lead to phosphorylation of IFNAR1 and subsequent recruitment of a E3 ligase that ubiquitinate IFNAR1. The KR mutant is completely resistant to ORF54-mediated degradation and the SR mutant is moderately susceptible. Compared to WT, there was no ubiquitination detected for the KR mutant and much reduced for the SR mutant in the presence of ORF54. The ubiquitination statuses of WT, KR and SR mutants correlate with their susceptibilities to ORF54-mediated degradation. From our previous result, we knew that ORF54 still induces the association of the KR mutant with UBAP1. This is the case for the SR mutant as well. Furthermore, we showed that ORF54 is able to increase serine phosphorylation of WT and the KR mutant. Based on this observation, we hypothesized that in the presence of ORF54, both of the KR and SR mutants enter the ESCRT pathway, but the KR mutant is not ubiquitinated and thus unable to be successfully shuttled into lysosomes.

Loss of HECTD1 reduces IFNAR1 expression.

We evaluated the function of HECTD1 in the ORF54-mediated mechanism of ORF54 IFNAR1 degradation. As a HECT-type E3 ligase interacting with ORF54, we hypothesized it facilitates the ubiquitination of IFNAR1 and thus its knockdown should rescue IFNAR1 expression in the presence of ORF54. To our surprise, knocking down HECT1 by siRNA further reduced IFNAR1 expression in both K5- and ORF54-transfected cells. This result reveals an unexpected role of HECTD1 in regulating IFNAR1 expression. Of note is that ORF54 does not impact the protein level of HECTD1.

To examine the interaction between ORF54 and HECTD1, we employed bimolecular fluorescence (Bi-FC). This assay employs a split fluorescent protein in order to measure associations within a short range. As the two halves are tagged onto proteins of interest, a short peptide linker connects the fluorescent halves from the enzymes under investigation. Cells are

transfected with the recombinant constructs and fluorescence measured compared to constructed negative controls[114]. For our purposes we employed the pBiFC-VN173 and pBiFC-VC155 expressing expressing fluorescent protein fragments derived from Venus BiFC. As negative control we employ the VC tagged fragment p22, a substrate of the SRC kinase. To evaluate HectD1 association with ORF54 we sub-cloned two of its protein interaction domains (HectD1-S1 and S2) and as control cloned the E3 ligase domain that exclusively binds ubiquitin. We inserted the HECTD1's protein interaction domains and E3 ligase domain into the Bi-FC expression vectors expressing split Venus and determined that ORF54 associates with the protein association domains, but fails to interact with the negative control fragment More experiments will need to investigate the association of HECTD1 and ORF54.

Discussion

Ubiquitination is important for the entry of internalized membrane proteins into the ESCRT machinery. After being captured by ESCRT, ubiquitination also plays a role in subsequent interactions with various components of ESCRT that contain ubiquitin-binding domains to ensure the passage to lysosomes. Therefore, it is not surprising that ORF54 does not completely eliminate the need of IFNAR1 ubiquitination for lysosomal degradation.

Previously we determined that the association between IFNAR1 and UBAP1 is independent of ubiquitin. Ligand-induced ubiquitination presumably takes place at the plasma membrane. Moreover, it has also been shown that ESCRT associates with multiple ubiquitin E3 ligases and deubiquitinases. If ubiquitination occurs to the cell surface-associated IFNAR1, it would promote IFNAR1 internalization. We did not observe the internalization rate of endogenous IFNAR being altered by ORF54. Thus, it is tempting to speculate that the enhanced ubiquitination of IFNAR1 takes place at the ESCRT machinery. The hypothesis remains preliminary as it is unclear how and where IFNAR1 ubiquitination takes place in the presence of ORF54. In the future, we will compare the impact of inhibiting endocytosis by CPZ on the ubiquitination status of IFNAR1 between IFNα treatment and ORF54 expression.

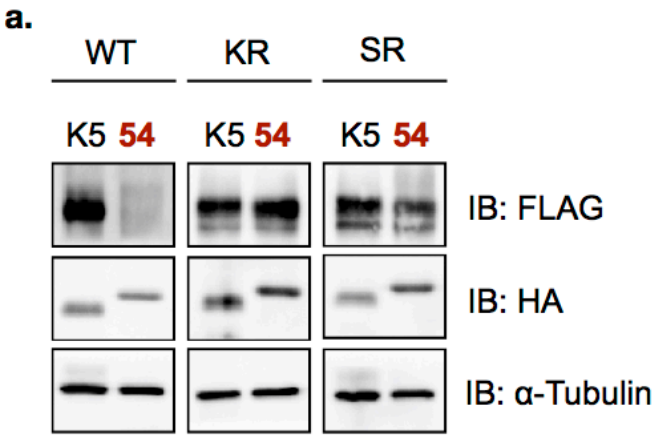
We showed that ORF54 induces less ubiquitination of the SR mutant than of WT. Phosphorylation at the serine residues 535 and 539 is important for the recruitment of SCF^{HOS} E3 ligase. This phosphorylation occurs to the cell surface-associated IFNAR1 and can be induced by several signaling pathways, including unfolded protein response (UPR) and p38 kinase pathway in addition to IFN signaling pathway. Exogenous expression of IFNAR1 triggers UPR [67], which leads to phosphorylation of IFNAR1 even without IFNα treatment and its subsequent ubiquitination. In the absence of ORF54, ubiquitination of exogenously expressed IFNAR1 does

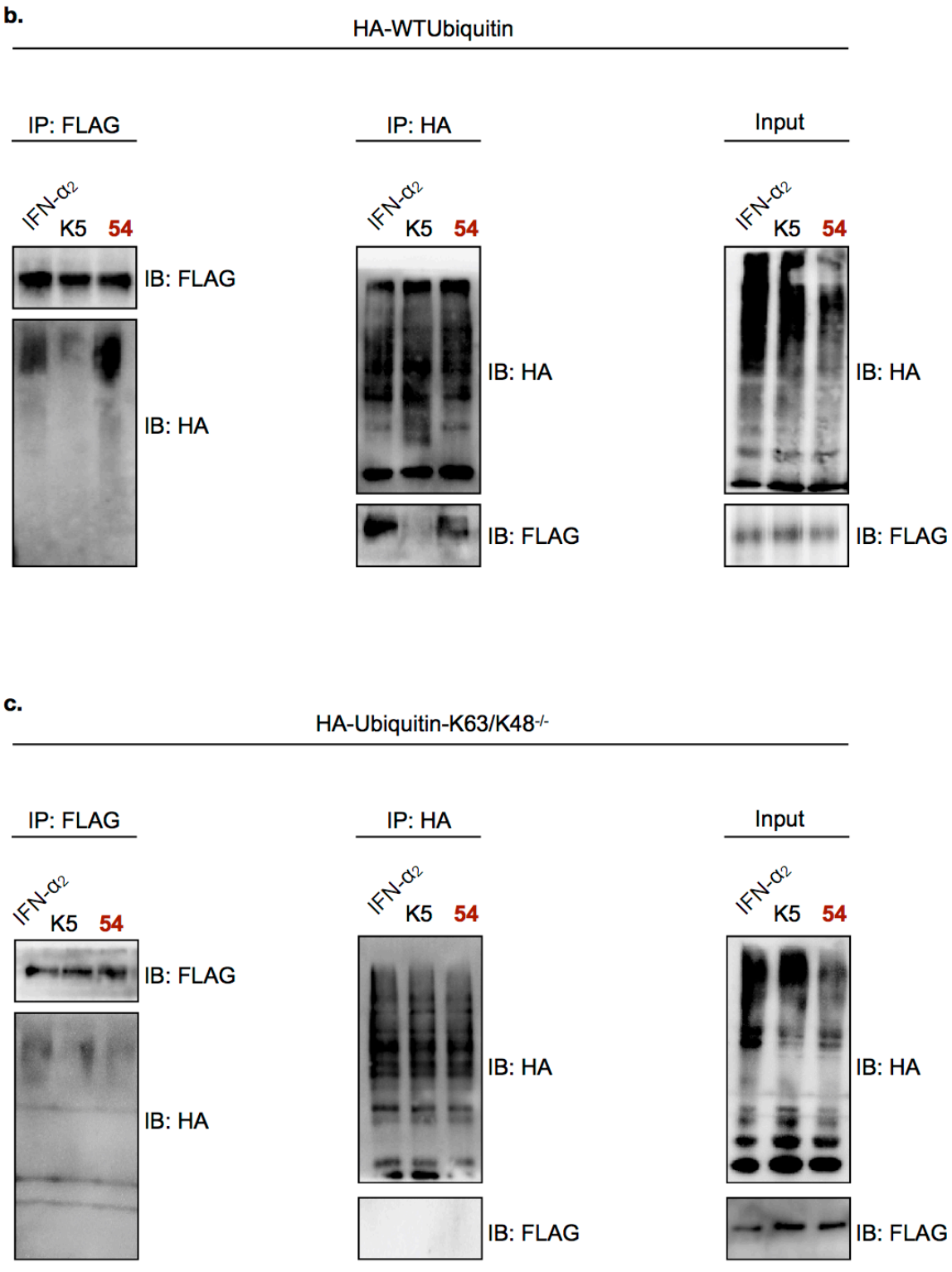
not occur to the SR mutant, indicating the dependence on phosphorylation. In the presence of ORF54, we observed some ubiquitination of the SR mutant, therefore degradation. We speculate that ORF54 facilitates the entry of the un-ubiquitinated SR mutant into the ESCRT machinery to allow for ubiquitination (Figure 4S.). For WT, ubiquitination can occur both through induction of phosphorylation at the cell surface and at ESCRT. As a result, there would be less ubiquitinated SR mutant compared to WT in the presence of ORF54. Similarly, there would be more ubiquitinated WT in the presence of ORF54 due to the enhanced sorting to ESCRT. To test this, CPZ can be employed to test whether the ORF54-mediated enhancement on ubiquitination is abolished by inhibiting endocytosis.

It is puzzling to detect enhanced phosphorylation of exogenously expressed IFNAR1 in the presence of ORF54. ORF54 enhances ubiquitination of endogenous IFNAR1 but not its phosphorylation (Figure 3-2i). One major difference between exogenously expressed and endogenous IFNAR1, as mentioned above, is the induction of UPR that leads phosphorylation of IFNAR1. Our result clearly showed that ORF54 does not induce any signaling event that causes phosphorylation of endogenous IFNAR1. Thus, we speculate that ORF54 inhibits dephosphorylation of IFNAR1 by trapping phosphorylated exogenously expressed IFNAR1 in the ESCRT machinery.

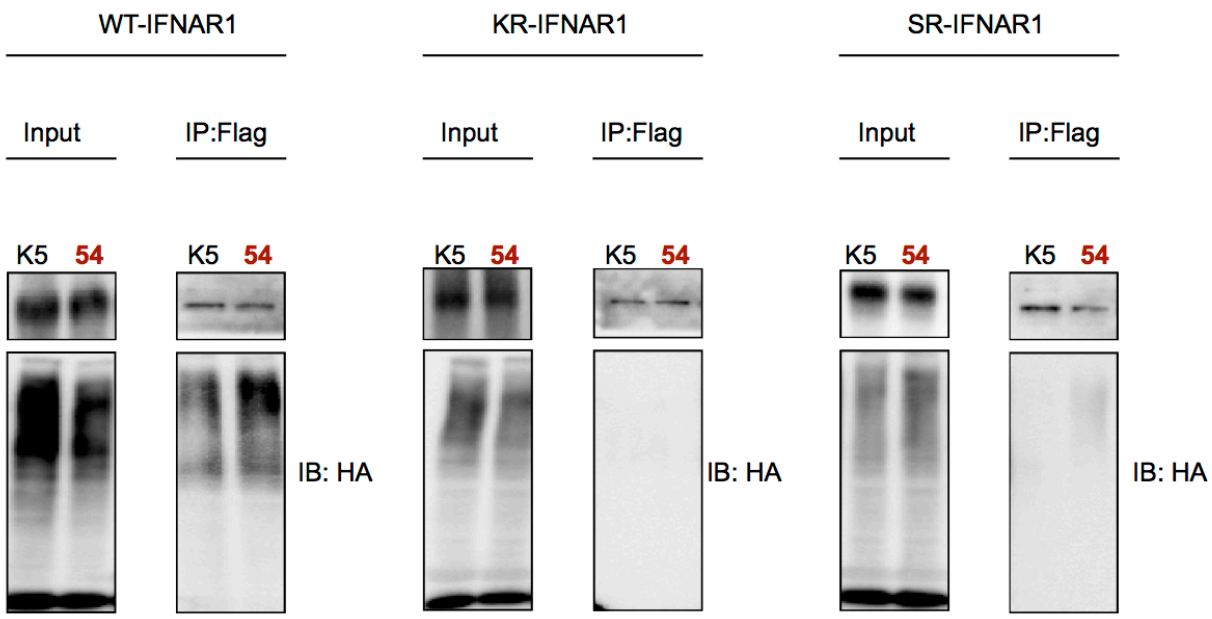
As an interaction partner of ORF54 identified in a previous Y2H screen, HECTD1 presents a viable candidate for the ubiquitination of IFNAR1. To our surprise, the data indicates that the loss of HECTD1 further reduces IFNAR1. Not much is known about the endogenous functions of HECTD1, and thus, its role in the mechanism of ORF54 remains most elusive. Clearly, ORF54 does not induce the degradation of HECTD1. This observation led us to propose that ORF54 may block the function of HECTD1 in regulating IFNAR1 expression. Previously we made a puzzling observation that MG132 treatment to inhibit proteosomal degradation also

further reduced IFNAR1 expression. We hypothesize that there may be relationship between these two observations and that HECTD1 may degrade a negative regulator of IFANR1 through proteasomes. This hypothesis is preliminary and require future studies to support. For example, we need to determine whether HECTD1 regulates IFNAR1 post-translationally and whether the E3 ligase activity is involved.

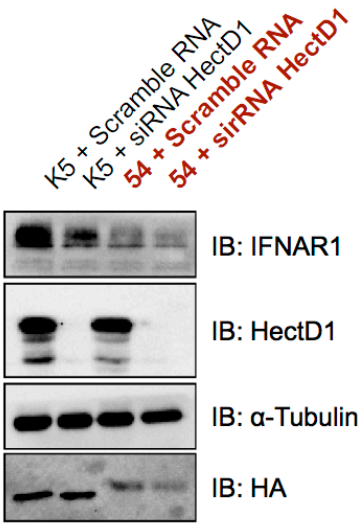

a.
WT
KR
SR
K5 54
K5 54
K5 54
IB: FLAG
IB: HA
IB: α-Tubulin


b.
HA-WTUbiquitin
IP: FLAG
IP: HA
Input
IFN-α2
K5
54
IB: FLAG
IB: HA
IB: HA
IB: FLAG
IB: HA
IB: FLAG
c.
HA-Ubiquitin-K63/K48-/-
IP: FLAG
IP: HA
Input
IFN-α2
K5
54
IB: FLAG
IB: HA
IB: HA
IB: FLAG
IB: HA
IB: FLAG

d.


WT-IFNAR1
KR-IFNAR1
SR-IFNAR1
Input
IP:Flag
Input
IP:Flag
Input
IP:Flag
K5 54
K5 54
K5 54
K5 54
K5 54
K5 54
IB: HA
IB: HA
IB: HA

e.


K5 + Scramble RNA
K5 + siRNA HectD1
54 + Scramble RNA
54 + siRNA HectD1
IB: IFNAR1
IB: HectD1
IB: α-Tubulin
IB: HA

f.

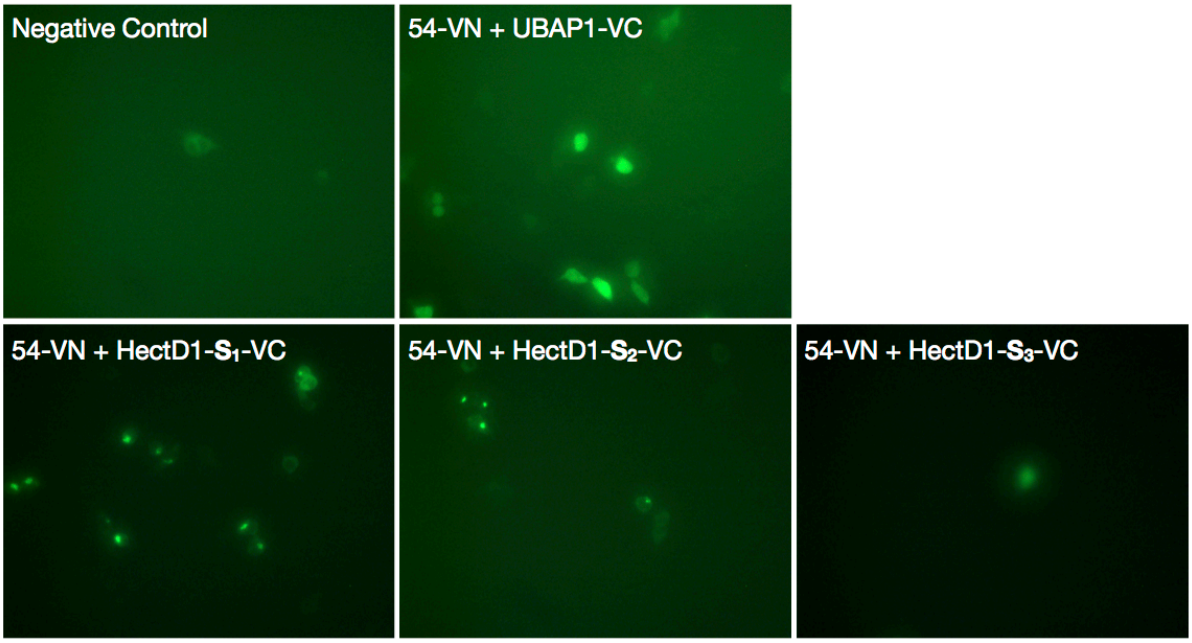

Negative Control
54-VN + UBAP1-VC
54-VN + HectD1-S1-VC
54-VN + HectD1-S2-VC
54-VN + HectD1-S3-VC

Figure 1. *ORF54-mediated degradation of IFNAR1 requires ubiquitination.* 293T cells expressing WT-IFNAR1, KR-IFNAR1, and SR-IFNAR1 display a targeted degradation of WT IFNAR1, a less significant down-regulation of SR-IFNAR1. Notably, the KR-IFNAR1 mutant remains immune to ORF54 degradation suggesting ubiquitination is required for IFNAR1 degradation (a). We also noted that WT-IFNAR1 is more highly ubiquitinated, and that ubiquitin chain propagation is likely dependent on K63/K48 ubiquitin linkage (b-c). 293T cells were used to KD HectD1 via siRNA and determine the impact of on IFNAR1 expression in the context of ORF54. We determined that KD of HectD1 down-regulates IFNAR1 independently of ORF54 and when combined further reduces receptor expression (d). We also confirmed that ORF54 associates with HectD1 protein association domains, as evidenced by the Bi-FC data in panel (f). As positive control for ORF54 association we employed UBAP1 as an interaction partner. We observed that HectD1 segments S1 and S2, interact with ORF54, while S3 (E3 ligase domain) does not (f).

Conclusions and Future Directions

Previous work established the viral dUTPase encoded by KSHV's ORF54 down-regulate the IFNAR1 protein resulting in the inhibition of the type-I IFN response. Lacking from prior studies is a clear understanding of ORF54's molecular mechanism of action. The emphasis of this dissertation, therefore, is the elucidation of molecular mechanism employed by ORF54 to degrade IFNAR1. Chapter Three outlines a detailed molecular and biochemical study of ORF54. We confirmed that KSHV's ORF54 promotes the degradation of IFNAR1 and gp130 in 293T cells and macrophages differentiated from monocytic U937 cells. We showed that ORF54 regulate IFNAR1 protein post-translationally, reducing its half-life. In addition to the loss of the total protein level, the surface expression of IFNAR1 is also largely reduced by ORF54, resulting in the functional inhibition of the type-I IFN response as measured by the induction of the ISG54. For all of these experiments, we included EGFR as a control for the selectivity of ORF54.

EGFR was chosen because ligand-induced EGFR degradation is mediated by UBAP1[80], a critical host factor for the mechanism of ORF54. In addition, we also included IFNAR2, the other subunit of the heteromeric IFNAR receptor. Presumably, IFNAR2 is not associated with IFNAR1 without IFNs and their association only occurs upon ligand binding. Collectively, the data indicate that the activity of ORF54 is not global but specific for a set of surface molecules. Missing from this analysis is the breath of proteins targeted by ORF54. As such, we propose a systems approach in the future to examine the scope of ORF54's on the proteome of the plasma membrane. We will perform the proteomics analysis using endothelial cells (dermal microvascular endothelial cell, DMVEC) and B-cells, the two biologically relevant cell types for KSHV pathogenesis. KSHV-associated malignancies are of B-cell and endothelial origins. Selective surface biotinylation followed by protein purification with streptavidin beads will be

carried out for subsequent mass spectrometry analysis. The surface proteome determines cellular responses to the extracellular environment. A comprehensive characterization of ORF54's targets would provide more in-depth insight towards the impact of on cell surface proteins, which may enable new therapeutic strategies against viral infections.

We also determined that ORF54 hijacks the ESCRT machinery to sort IFNAR1 towards the lysosome. Chemical inhibition of the lysosome and genetic inhibition of ESCRT-mediated protein sorting results in the rescue of IFNAR1 and reconstitution of the type-I IFN response in the presence of ORF54. Actively missing from this investigation is how ORF54 achieves selectivity. After constitutive internalization, receptors are recycled or down-regulated receptors through their respective trafficking routes[109]. Ubiquitin modifications in the cytoplasmic domains of cargo proteins are the major sorting signal for ESCRT[81-82,109]. About 50% of EGFR is internalized within an hour (Fig. 3-2h). Thus, the absence of EGFR's degradation in the presence of ORF54 is not due to the lack of internalized EGFR.

We showed that ORF54 enables the interaction of endocytosed IFNAR1 and UBAP1, a subunit of ESCRT-I, independent of ubiquitination. This ORF54-mediated interaction was not found between EGFR and UBAP1, despite that UBAP1 is essential for ligand-induced ubiquitin-dependent EGFR degradation. It is unclear whether this occurs because internalized EGFR is actively recycled to the surface rather than just via passive membrane flow and thus, does not dwell on the endosome long enough for ORF54 to grab. Alternatively, a specific domain at the cytoplasmic tail of a receptor provide sequence-specific, direct or indirect, interactions with ORF54. We did observe ORF54's specific association with IFNAR1 and not with EGFR. This association is independent of UBAP1 but requires endocytosis. Therefore, we conclude that ORF54 associates with its targets after they endocytosed. However, the molecular details of this association remain to be determined.

For future investigations, we will employ CD4 chimera by fusing the cytoplasmic tail of IFNAR1 or EGFR with the extracellular and transmembrane part of CD4. The CD4 chimera system has been extensively used to study the endocytic sorting signals in a given cytoplasmic tail. We will determine whether the interaction between ORF54 and its targets is direct or indirect via a cellular protein. ORF54 provides a novel model to study endocytic trafficking of cytokine receptors. The decision of whether an internalized receptor molecule is recycled or degraded is a major mechanism to regulate its level at the cell surface which determines the strength and duration of ligand induced signaling cascades. Much attention has been given to ligand induced regulation, and yet, very little is known about basal recycling of cytokine receptors, including IFNAR1 and gp130.

We determined that UBAP1 is an essential host factor for ORF54-mediated IFNAR1 down-regulation. The interaction with UBAP1 was identified through yeast two hybrid screening, indicating a direct physical interaction. The ability of ORF54 to interact with UBAP1 allows it to funnel endocytosed proteins not designated for degradation to the ESCRT machinery and subsequently to lysosomes[80,89]. In addition, the yeast two hybrid screening identified other potential cellular interacting proteins of ORF54, such as BAT2D1 (BAT 2 domain-containing protein 1), HECTD1 (HECT domain-containing protein), SSFA2 (sperm specific antigen 2), and WNK1 (With-No-Lysine kinase 1). Their roles in ORF54-mediated IFNAR1 degradation remains to be determined. Our study indicates that ubiquitination of IFNAR1 is likely not needed for sorting to the ESCRT machinery but is still required for degradation.

HECTD1, a E3 ligase, seems to be a viable candidate protein contributing to IFNAR1 ubiquitination. In contrast, we showed that that knocking down HECTD1 alone causes the degradation of IFNAR1. This result indicates that HECTD1 positively regulates IFNAR1 expression, which is an exciting phenotype on its own. Based on this observation, we

hypothesized that ORF54 inhibits the function of HECTD1 to destabilize IFNAR1. The limited amount of information available regarding this E3 ligase make it difficult to hypothesize possible underlying molecular mechanisms. Furthermore, it remains to be determined whether the E3 ligase function of HECTD1 is required for its role in regulating IFNAR1 expression and whether this regulation occurs at the post-translational level. While unclear, research evaluating this mechanism may yield potential therapeutic targets limiting IFN signals in some autoimmune diseases.

It is puzzling to observe that ORF54 bypasses the requirement of ESCRT-0. ESCRT-I has only weak affinity for early endosomal membranes. HRS, a critical component of ESCRT-0, has high affinity for a specific phospholipid, PI3P, enriched in early endosomes[83]. Therefore, it is thought that endosome-associated ESCRT-0 initiates the sorting by seizing ubiquitinated proteins and recruits ESCRT-I. While ORF54 circumvents the need of ubiquitination for sorting to the ESCRT machinery, ESCRT-I still needs ESCRT-0 to be localized at the endosome. And yet, knocking down HRS does not rescue IFNAR1 expression in the presence of ORF54. One possibility is that alternative ESCRT-0 complexes recruit ESCRT-I to the early endosomes. Another is that an additional cellular interacting protein of ORF54 anchor ESCRT-I to the early endosomes.

ORF54 appears to localize in the early and late endosomal compartments. What determines the localization of ORF54 in the endosome remains to be determined. It is tempting to ascribe this localization to the association with UBAP1, as the ORF54 mutants that fail to interact with UBAP1 also lose its endosomal localization. However, if endosomal localization of UBAP1 depends on recruitment by ESCRT-0, the interaction with UBAP1 still does not explain why ORF54-mediated IFNAR1 degradation occurs independent of ESCRT-0. In the future, we will examine the role of HRS and ORF54 in the localization of ESCRT-I.

We demonstrated the function of ORF54 in down-regulating IFNAR1 and type-I IFN responses during KSHV lytic replication. Notably ORF54's function is time-specific, because at later time points IFNAR1 is degraded regardless of whether ORF54 is expressed or not. This is not surprising and indicates that KSHV have evolved multiple mechanisms to evade type-I IFN responses. While in MHV-68 ORF54 is expressed as an early gene, we still need to determine ORF54's expression kinetics. To this end, we propose the construction of a FLAG- tagged ORF54 KSHV as well as generate ORF54-specific antibodies. Including K3 and K5, KSHV encodes at least three lytic genes to modulate expression of surface molecules. A comprehensive understanding of alterations on the surface proteomes during KSHV lytic replication and latent infection is still lacking. In the future, we will employ selective surface biotinylation combined with MS to study how KSHV reprograms the cell surface. This information is likely to provide therapeutic targets specific for KSHV-infected cells.

Our study has uncovered a novel, evolutionarily conserved mechanism by which herpesviruses counteract IFN-I responses. We showed that herpesviral dUTPases interact with UBAP1 and down-regulate IFNAR1, a function independent of its nucletotide hydrolyzing activity and absent from cellular counterparts[66]. Using KSHV, we further demonstrated the biological significance of this anti-IFN mechanism during viral replication. This thesis research has opened a new avenue for future explorations of how endocytic trafficking of cytokine receptors is regulated and strategies that can limit viral infection and its pathogenic consequence.

We know from the MHV68 studies that ORF54 supports the efficient establishment of viral latency. We hypothesized that virus lacking ORF54 is sensitive to the type-I IFN response. Our data suggest that 54S-KSHV loses the ability to degrade IFNAR1 during lytic infection efficiently, leading to an enhanced type-I IFN response. Furthermore, the resulting antiviral response appears to limit viral replication in the absence of ORF54.. Investigating ORF54's

function in vivo requires the use of MHV-68. To that end, we propose to construct MHV-68 clones containing ORF54 loss of function mutants, instead of completely limiting the expression of ORF54 as in previous work. Furthermore, missing from this work is an understanding of ORF54's expression dynamics. To evaluate ORF54's expression, we propose the construction of a tagged ORF54 KSHV. The power of this approach will allow us to evaluate ORF54's function fully supported by the viral infection. It may be worth evaluating ORF54's impact on the cell membrane proteome in this context.

Chapter Three provides some preliminary data investigating the role of ubiquitination of IFNAR1. Following the observation that KR-IFNAR1 is immune to ORF54's degradation, we hypothesized that ubiquitination is essential to IFNAR1's degradation. Indeed, we observe a significant increase of ubiquitinated IFNAR1 in the presence of ORF54. It remains unclear whether ORF54 recruits adaptor proteins to influence INFAR1's degradation. It is also unknown to us if IFNAR1 is ubiquitinated before entry into the ESCRT-pathway.

ORF54's influence on ubiquitination is also interesting. It is also unclear whether ORF54 universally requires ubiquitination to occur for the degradation of all targeted species. A detailed study of the ubiquitin profile in the presence of ORF54 would provide critical insight into this question.

In conclusion, we defined a molecular mechanism by which KSHV's ORF54 antagonizes the type-I IFN response by selectively targeting IFNAR1 towards lysosomal degradation. Based on our evidence, we propose a model mechanism that enhances the degradation of constitutively endocytosed IFNAR1 in the presence of ORF54. Further research evaluating ORF54's selectivity, the influence on the ubiquitin-independent association with UBAP1, and the scope of targeted receptor species are needed to comprehend the impact of this viral dUTPase.

Supplemental Data

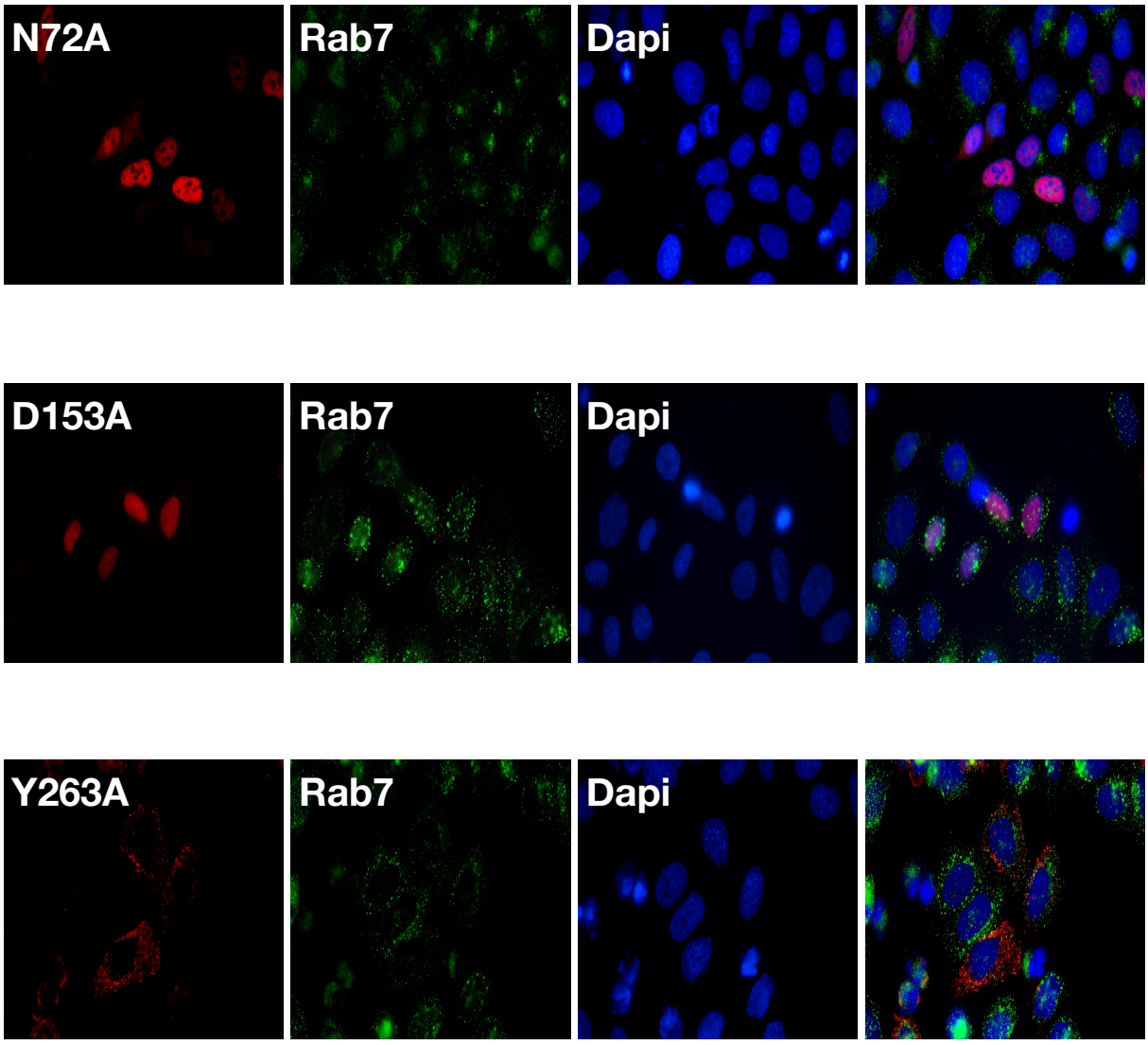

N72A
Rab7
Dapi
D153A
Rab7
Dapi
Y263A
Rab7
Dapi

Figure 1S. *ORF54 loss of function mutants lost localization in the endosome.* Mutants N72 and D153 localize in the nucleus, while Y263 is in the cytoplasm but loses association with the endosomal vescicle.

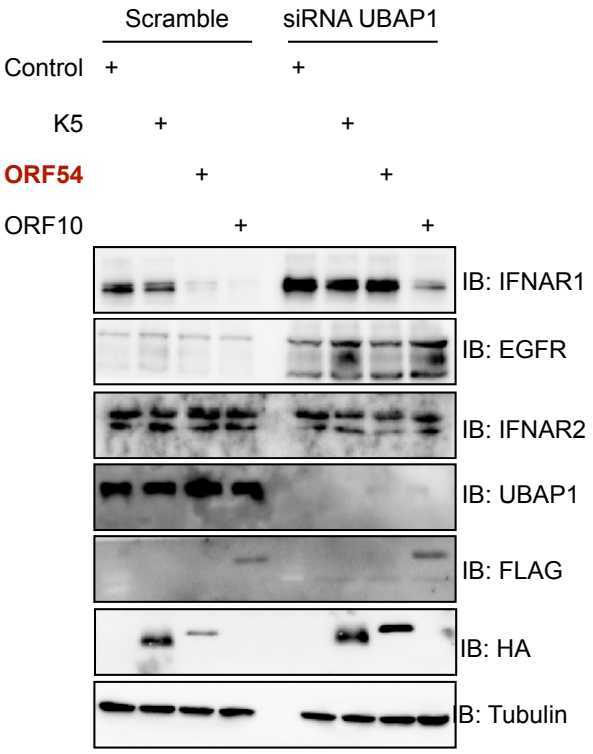

Scramble
siRNA UBAP1
Control + +
K5 + +
ORF54 + +
ORF10 + +
IB: IFNAR1
IB: EGFR
IB: IFNAR2
IB: UBAP1
IB: FLAG
IB: HA
B: Tubulin

Figure 2S. *KD of UBAP1 rescues IFNAR1in the presence of ORF54.* To evaluate UBAP1's functional relevance we performed siRNA KD experiments and determined that loss of UBAP1 rescues IFNAR1 in the presence of ORF54 as well as upregulates IFNAR1 in K5 treated cells. Of note is the phenotype in ORF10 transfected cells. ORF10 inhibits IFNAR1 via an independent mechanism that is not inhibited by the loss of IFNAR1, thus we observe only a mild upregulation of the receptor subunit in ORF10 positive cells.

a.

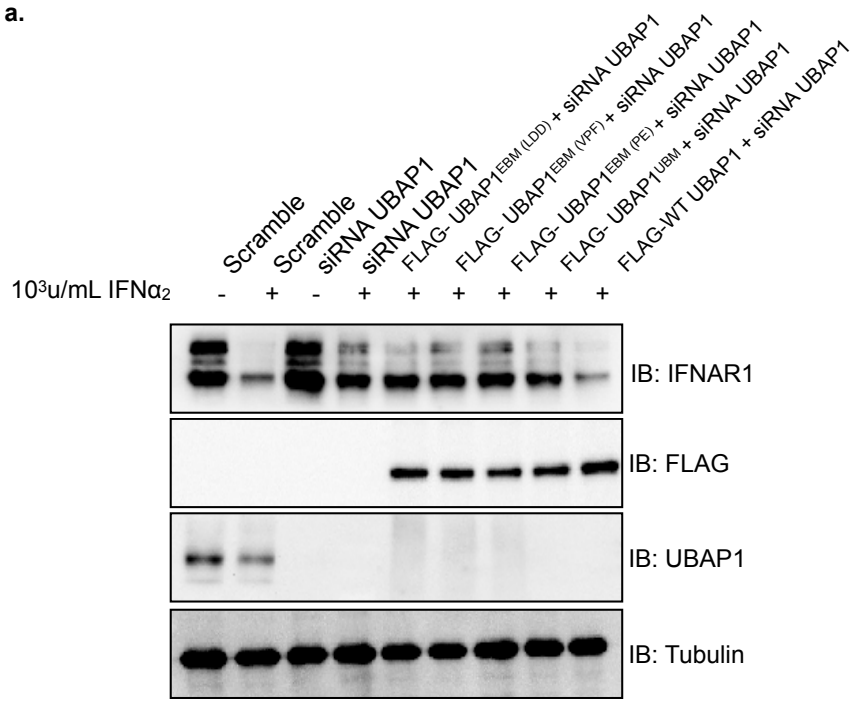

Scramble
Scramble
siRNA UBAP1
siRNA UBAP1
FLAG- UBAP1EBM (LDD) + siRNA UBAP1
FLAG- UBAP1EBM (VPF) + siRNA UBAP1
FLAG- UBAP1EBM (PE) + siRNA UBAP1
FLAG- UBAP1UBM + siRNA UBAP1
FLAG-WT UBAP1 + siRNA UBAP1
10³u/mL IFNα2 - + - + + + + + +
IB: IFNAR1
IB: FLAG
IB: UBAP1
IB: Tubulin

Figure 3A. *IFN-alpah2 requires UBAP1's ubiquitin binding function to induce IFNAR1 degradation.* In contrast, IFN-α requires both ESCRT-binding and UBAP1-binding to facilitate the ligand-induced degradation of IFNAR1.

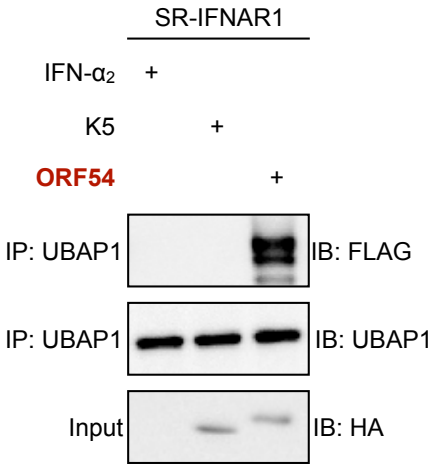

SR-IFNAR1
IFN-α2 +
K5 +
ORF54 +
IP: UBAP1
IB: FLAG
IP: UBAP1
IB: UBAP1
Input
IB: HA

Figure 4S. We overexpressed the SR-IFNAR1 mutant and ORF54. We then IPed UBAP1 and determined that in the presence of ORF54, the SR-IFNAR1 mutant maintains an association with UBAP1. This observation is in contrast to IFN-α treatment, mimicking the results of KR-IFNAR1.

REFERENCES

1. Sun, R., Lin, S., Gradoville, L., Yuan, Y., Zhu, F., & Miller, G. (1998). A viral gene that activates lytic cycle expression of Kaposis sarcoma-associated herpesvirus. *Proceedings of the National Academy of Sciences,95*(18), 10866-10871. doi:10.1073/pnas.95.18.10866

2. Lukac, D. M., Renne, R., Kirshner, J. R., & Ganem, D. (1998). Reactivation of Kaposis Sarcoma-Associated Herpesvirus Infection from Latency by Expression of the ORF 50 Transactivator, a Homolog of the EBV R Protein. *Virology,252*(2), 304-312. doi:10.1006/viro.1998.9486

3. Hwang, S., Wu, T., Tong, L. M., Kim, K. S., Martinez-Guzman, D., Colantonio, A. D., Sun, R. (2008). Persistent Gammaherpesvirus Replication and Dynamic Interaction with the Host In Vivo. *Journal of Virology,82*(24), 12498-12509. doi:10.1128/jvi.01152-08

4. Gradoville, L., Gerlach, J., Grogan, E., Shedd, D., Nikiforow, S., Metroka, C., & Miller, G. (2000). Kaposis Sarcoma-Associated Herpesvirus Open Reading Frame 50/Rta Protein Activates the Entire Viral Lytic Cycle in the HH-B2 Primary Effusion Lymphoma Cell Line. *Journal of Virology,74*(13), 6207-6212. doi:10.1128/jvi.74.13.6207-6212.2000

5. Bechtel, J. T., Winant, R. C., & Ganem, D. (2005). Host and Viral Proteins in the Virion of Kaposis Sarcoma-Associated Herpesvirus. *Journal of Virology,79*(8), 4952-4964. doi:10.1128/jvi.79.8.4952-4964.2005

6. Cesarman, E. (2011). Gammaherpesvirus and lymphoproliferative disorders in immunocompromised patients. *Cancer Letters,305*(2), 163-174. doi:10.1016/j.canlet.2011.03.003

7. Coscoy, L. (2007). Immune evasion by Kaposis sarcoma-associated herpesvirus. *Nature Reviews Immunology,7*(5), 391-401. doi:10.1038/nri2076

8. Feng, P., Moses, A., & Früh, K. (2013). Evasion of adaptive and innate immune response mechanisms by γ-herpesviruses. *Current Opinion in Virology,3*(3), 285-295. doi:10.1016/j.coviro.2013.05.011

9. Lee, H., Lee, S., Chaudhary, P. M., Gill, P., & Jung, J. U. (2010). Immune evasion by Kaposi's sarcoma-associated herpesvirus. *Future Microbiology,5*(9), 1349-1365. doi:10.2217/fmb.10.105

10. Paludan, S. R., Bowie, A. G., Horan, K. A., & Fitzgerald, K. A. (2013). Recognition of herpesviruses by the innate immune system. *Nature Reviews Immunology,13*(8), 614-614. doi:10.1038/nri3497

11. Sathish, N., & Yuan, Y. (2011). Evasion and Subversion of Interferon-Mediated Antiviral Immunity by Kaposis Sarcoma-Associated Herpesvirus: An Overview. *Journal of Virology,85*(21), 10934-10944. doi:10.1128/jvi.00687-11

12. Wei, X., & Lan, K. (2018). Activation and counteraction of antiviral innate immunity by KSHV: An update. *Science Bulletin,63*(18), 1223-1234. doi:10.1016/j.scib.2018.07.009

13. Kumar, B., & Chandran, B. (2016). KSHV Entry and Trafficking in Target Cells—Hijacking of Cell Signal Pathways, Actin and Membrane Dynamics. *Viruses,8*(11), 305. doi:10.3390/v8110305

14. Pertel, P. E. (2002). Human Herpesvirus 8 Glycoprotein B (gB), gH, and gL Can Mediate Cell Fusion. *Journal of Virology,76*(9), 4390-4400. doi:10.1128/jvi.76.9.4390-4400.2002

15. Birkmann, A., Mahr, K., Ensser, A., Yaguboglu, S., Titgemeyer, F., Fleckenstein, B., & Neipel, F. (2001). Cell Surface Heparan Sulfate Is a Receptor for Human Herpesvirus 8 and Interacts with Envelope Glycoprotein K8.1. *Journal of Virology,75*(23), 11583-11593. doi:10.1128/jvi.75.23.11583-11593.2001

16. Wang, F., Akula, S. M., Sharma-Walia, N., Zeng, L., & Chandran, B. (2003). Human Herpesvirus 8 Envelope Glycoprotein B Mediates Cell Adhesion via Its RGD Sequence. *Journal of Virology,77*(5), 3131-3147. doi:10.1128/jvi.77.5.3131-3147.2003

17. Akula, S. M., Wang, F., Vieira, J., & Chandran, B. (2001). Human Herpesvirus 8 Interaction with Target Cells Involves Heparan Sulfate. *Virology,282*(2), 245-255. doi:10.1006/viro.2000.0851

18. Kaleeba, J. A. (2006). Kaposis Sarcoma-Associated Herpesvirus Fusion-Entry Receptor: Cystine Transporter xCT. *Science,311*(5769), 1921-1924. doi:10.1126/science.1120878

19. Lan, K., Kuppers, D. A., Verma, S. C., & Robertson, E. S. (2004). Kaposis Sarcoma-Associated Herpesvirus-Encoded Latency-Associated Nuclear Antigen Inhibits Lytic Replication by Targeting Rta: A Potential Mechanism for Virus-Mediated Control of Latency. *Journal of Virology,78*(12), 6585-6594. doi:10.1128/jvi.78.12.6585-6594.2004

20. Chang, Y., Moore, P. S., Talbot, S. J., Boshoff, C. H., Zarkowska, T., Godden-Kent, D., . . . Mittnacht, S. (1996). Cyclin encoded by KS herpesvirus. *Nature,382*(6590), 410-410. doi:10.1038/382410a0

21. Guasparri, I., Keller, S. A., & Cesarman, E. (2004). KSHV vFLIP Is Essential for the Survival of Infected Lymphoma Cells. *The Journal of Experimental Medicine,199*(7), 993-1003. doi:10.1084/jem.20031467

22. Dedicoat, M., Newton, R., (2003). Review of the distribution of Kaposi's sarcoma-associated herpesvirus (KSHV) in Africa in relation to the incidence of Kaposi's sarcoma. *The British Journal of Cancer*, 88, 1-3

23. Kumar, B., Dutta, D., Iqbal, J., Ansari, M. A., Roy, A., Chikoti, L., Chandran, B. (2016). ESCRT-I Protein Tsg101 Plays a Role in the Post-macropinocytic Trafficking and Infection of Endothelial Cells by Kaposi's Sarcoma-Associated Herpesvirus. *PLOS Pathogens,12*(10). doi:10.1371/journal.ppat.1005960

24. Uematsu, S., & Akira, S. (2006). Innate Immune recognition of viral infection. *Uirusu,56*(1), 1-8. doi:10.2222/jsv.56.1

25. Crawford, A., & Wherry, E. J. (2009). The diversity of costimulatory and inhibitory receptor pathways and the regulation of antiviral T cell responses. *Current Opinion in Immunology,21*(2), 179-186. doi:10.1016/j.coi.2009.01.010

26. Akira, S. (n.d.). TLR Signaling. *Current Topics in Microbiology and Immunology From Innate Immunity to Immunological Memory,*1-16. doi:10.1007/3-540-32636-7_1

27. Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen Recognition and Innate Immunity. *Cell,124*(4), 783-801. doi:10.1016/j.cell.2006.02.015

28. Beutler, B. A. (2008). TLRs and innate immunity. *Blood,113*(7), 1399-1407. doi:10.1182/blood-2008-07-019307

29. West, J., & Damania, B. (2008). Upregulation of the TLR3 Pathway by Kaposis Sarcoma-Associated Herpesvirus during Primary Infection. *Journal of Virology,82*(11), 5440-5449. doi:10.1128/jvi.02590-07

30. Lagos, D., Vart, R. J., Gratrix, F., Westrop, S. J., Emuss, V., Wong, P., Boshoff, C. (2008). Toll-like Receptor 4 Mediates Innate Immunity to Kaposi Sarcoma Herpesvirus. *Cell Host & Microbe,4*(5), 470-483. doi:10.1016/j.chom.2008.09.012

31. Ahmad, H., Gubbels, R., Ehlers, E., Meyer, F., Waterbury, T., Lin, R., & Zhang, L. (2011). Kaposi Sarcoma-associated Herpesvirus Degrades Cellular Toll-Interleukin-1 Receptor Domain-containing Adaptor-inducing β-Interferon (TRIF). *Journal of Biological Chemistry,286*(10), 7865-7872. doi:10.1074/jbc.m110.191452

32. Bussey, K. A., Reimer, E., Todt, H., Denker, B., Gallo, A., Konrad, A., Brinkmann, M. M. (2014). The Gammaherpesviruses Kaposis Sarcoma-Associated Herpesvirus and Murine Gammaherpesvirus 68 Modulate the Toll-Like Receptor-Induced Proinflammatory Cytokine Response. *Journal of Virology,88*(16), 9245-9259. doi:10.1128/jvi.00841-14

33. Meyer, F., Ehlers, E., Steadman, A., Waterbury, T., Cao, M., & Zhang, L. (2013). TLR-TRIF Pathway Enhances the Expression of KSHV Replication and Transcription Activator. *Journal of Biological Chemistry,288*(28), 20435-20442. doi:10.1074/jbc.m113.487421

34. Zhao, Q., Liang, D., Sun, R., Jia, B., Xia, T., Xiao, H., & Lan, K. (2014). Kaposis Sarcoma-Associated Herpesvirus-Encoded Replication and Transcription Activator Impairs Innate Immunity via Ubiquitin-Mediated Degradation of Myeloid Differentiation Factor 88. *Journal of Virology,89*(1), 415-427. doi:10.1128/jvi.02591-14

35. Abend, J. R., Ramalingam, D., Kieffer-Kwon, P., Uldrick, T. S., Yarchoan, R., & Ziegelbauer, J. M. (2012). Kaposis Sarcoma-Associated Herpesvirus MicroRNAs Target IRAK1 and MYD88, Two Components of the Toll-Like Receptor/Interleukin-1R Signaling Cascade, To Reduce Inflammatory-Cytokine Expression. *Journal of Virology,86*(21), 11663-11674. doi:10.1128/jvi.01147-12

36. Inn, K., Lee, S., Rathbun, J. Y., Wong, L., Toth, Z., Machida, K., Jung, J. U. (2011). Inhibition of RIG-I-Mediated Signaling by Kaposis Sarcoma-Associated Herpesvirus-Encoded Deubiquitinase ORF64. *Journal of Virology,85*(20), 10899-10904. doi:10.1128/jvi.00690-11

37. West, J. A., Wicks, M., Gregory, S. M., Chugh, P., Jacobs, S. R., Zhang, Z., . . . Damania, B. (2014). An Important Role for Mitochondrial Antiviral Signaling Protein in the Kaposis Sarcoma-Associated Herpesvirus Life Cycle. *Journal of Virology,88*(10), 5778-5787. doi:10.1128/jvi.03226-13

38. Gregory, S. M., Davis, B. K., West, J. A., Taxman, D. J., Matsuzawa, S., Reed, J. C., Damania, B. (2011). Discovery of a Viral NLR Homolog that Inhibits the Inflammasome. *Science,331*(6015), 330-334. doi:10.1126/science.1199478

39. Kerur, N., Veettil, M., Sharma-Walia, N., Bottero, V., Sadagopan, S., Otageri, P., & Chandran, B. (2011). IFI16 Acts as a Nuclear Pathogen Sensor to Induce the Inflammasome in Response to Kaposi Sarcoma-Associated Herpesvirus Infection. *Cell Host & Microbe,9*(5), 363-375. doi:10.1016/j.chom.2011.04.008

40. Baresova, P., Pitha, P. M., & Lubyova, B. (2013). Distinct Roles of Kaposis Sarcoma-Associated Herpesvirus-Encoded Viral Interferon Regulatory Factors in Inflammatory Response and Cancer. *Journal of Virology,87*(17), 9398-9410. doi:10.1128/jvi.03315-12

41. Jacobs, S. R., & Damania, B. (2011). The Viral Interferon Regulatory Factors of KSHV: Immunosuppressors or Oncogenes? *Frontiers in Immunology,2*. doi:10.3389/fimmu.2011.00019

42. Park, J., Lee, M., Yoo, S., Jeong, K. W., Lee, D., Choe, J., & Seo, T. (2007). Identification of the DNA Sequence Interacting with Kaposis Sarcoma-Associated Herpesvirus Viral Interferon Regulatory Factor 1. *Journal of Virology,81*(22), 12680-12684. doi:10.1128/jvi.00556-07

43. Seo, T., Lee, D., Lee, B., Chung, J. H., & Choe, J. (2000). Viral Interferon Regulatory Factor 1 of Kaposis Sarcoma-Associated Herpesvirus (Human Herpesvirus 8) Binds to, and Inhibits Transactivation of, CREB-Binding Protein. *Biochemical and Biophysical Research Communications,270*(1), 23-27. doi:10.1006/bbrc.2000.2393

44. Lin, R., Genin, P., Mamane, Y., Sgarbanti, M., Battistini, A., Harrington, W. J., Hiscott, J. (2001). HHV-8 encoded vIRF-1 represses the interferon antiviral response by blocking IRF-3 recruitment of the CBP/p300 coactivators. *Oncogene,20*(7), 800-811. doi:10.1038/sj.onc.1204163

45. Fuld, S., Cunningham, C., Klucher, K., Davison, A. J., & Blackbourn, D. J. (2006). Inhibition of Interferon Signaling by the Kaposis Sarcoma-Associated Herpesvirus Full-Length Viral Interferon Regulatory Factor 2 Protein. *Journal of Virology,80*(6), 3092-3097. doi:10.1128/jvi.80.6.3092-3097.2006

46. Mutocheluh, M., Hindle, L., Areste, C., Chanas, S. A., Butler, L. M., Lowry, K., Blackbourn, D. J. (2011). Kaposis sarcoma-associated herpesvirus viral interferon regulatory factor-2 inhibits type 1 interferon signalling by targeting interferon-stimulated gene factor-3. *Journal of General Virology,92*(10), 2394-2398. doi:10.1099/vir.0.034322-0

47. Burysek, L., & Pitha, P. M. (2001). Latently Expressed Human Herpesvirus 8-Encoded Interferon Regulatory Factor 2 Inhibits Double-Stranded RNA-Activated Protein Kinase. *Journal of Virology,75*(5), 2345-2352. doi:10.1128/jvi.75.5.2345-2352.2001

48. Damania, B. (2009). Faculty of 1000 evaluation for Identification of caspase-mediated decay of interferon regulatory factor-3, exploited by a Kaposi sarcoma-associated herpesvirus immunoregulatory protein. *F1000 - Post-publication Peer Review of the Biomedical Literature*. doi:10.3410/f.1161472.623008

49. Cloutier, N., & Flamand, L. (2010). Kaposi Sarcoma-associated Herpesvirus Latency-associated Nuclear Antigen Inhibits Interferon (IFN) β Expression by Competing with IFN Regulatory Factor-3 for Binding toIFNBPromoter. *Journal of Biological Chemistry,285*(10), 7208-7221. doi:10.1074/jbc.m109.018838

50. Moore, P. S. (2009). Faculty of 1000 evaluation for Kaposis sarcoma-associated herpesvirus viral interferon regulatory factor 4 targets MDM2 to deregulate the p53 tumor suppressor pathway. *F1000 - Post-publication Peer Review of the Biomedical Literature*. doi:10.3410/f.1159196.619518

51. Jacobs, S. R., Gregory, S. M., West, J. A., Wollish, A. C., Bennett, C. L., Blackbourn, D. J., Damania, B. (2012). The Viral Interferon Regulatory Factors of Kaposis Sarcoma-Associated

Herpesvirus Differ in Their Inhibition of Interferon Activation Mediated by Toll-Like Receptor 3. *Journal of Virology,87*(2), 798-806. doi:10.1128/jvi.01851-12

52. Lefort, S., Soucy-Faulkner, A., Grandvaux, N., & Flamand, L. (2007). Binding of Kaposis Sarcoma-Associated Herpesvirus K-bZIP to Interferon-Responsive Factor 3 Elements Modulates Antiviral Gene Expression. *Journal of Virology,81*(20), 10950-10960. doi:10.1128/jvi.00183-07

53. Coscoy, L., & Ganem, D. (2000). Kaposis sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis. *Proceedings of the National Academy of Sciences,97*(14), 8051-8056. doi:10.1073/pnas.140129797

54. Sanchez, D. J., Coscoy, L., & Ganem, D. (2001). Functional Organization of MIR2, a Novel Viral Regulator of Selective Endocytosis. *Journal of Biological Chemistry,277*(8), 6124-6130. doi:10.1074/jbc.m110265200

55. Li, Q., Means, R., Lang, S., & Jung, J. U. (2006). Downregulation of Gamma Interferon Receptor 1 by Kaposis Sarcoma-Associated Herpesvirus K3 and K5. *Journal of Virology,81*(5), 2117-2127. doi:10.1128/jvi.01961-06

56. Mansouri, M., Viswanathan, K., Douglas, J. L., Hines, J., Gustin, J., Moses, A. V., & Fruh, K. (2009). Molecular Mechanism of BST2/Tetherin Downregulation by K5/MIR2 of Kaposis Sarcoma-Associated Herpesvirus. *Journal of Virology,83*(19), 9672-9681. doi:10.1128/jvi.00597-09

57. Ishido, S., Wang, C., Lee, B., Cohen, G. B., & Jung, J. U. (2000). Downregulation of Major Histocompatibility Complex Class I Molecules by Kaposis Sarcoma-Associated Herpesvirus K3 and K5 Proteins. *Journal of Virology,74*(11), 5300-5309. doi:10.1128/jvi.74.11.5300-5309.2000

58. Lehner, P. J., Hoer, S., Dodd, R., & Duncan, L. M. (2005). Downregulation of cell surface receptors by the K3 family of viral and cellular ubiquitin E3 ligases. *Immunological Reviews,207*(1), 112-125. doi:10.1111/j.0105-2896.2005.00314.x

59. Stevenson, P., May, J., Smith, X., Marques, S., Adler, H., Koszinowski, U., Efstathiou, S. (2002). K3-mediated evasion of CD8 T cells aids amplification of a latent γ-herpesvirus. *Nature Immunology,3*(8), 733-740. doi:10.1038/ni818

60. Coscoy, L., & Ganem, D. (2001). A viral protein that selectively downregulates ICAM-1 and B7-2 and modulates T cell costimulation. *Journal of Clinical Investigation,107*(12), 1599-1606. doi:10.1172/jci12432

61. Thomas, M., Wills, M., & Lehner, P. (2008). Natural killer cell evasion by an E3 ubiquitin ligase from Kaposis sarcoma-associated herpesvirus. *Biochemical Society Transactions,36*(3), 459-463. doi:10.1042/bst0360459

62. Karki, R., Lang, S. M., & Means, R. E. (2011). The MARCH Family E3 Ubiquitin Ligase K5 Alters Monocyte Metabolism and Proliferation through Receptor Tyrosine Kinase Modulation. *PLoS Pathogens,7*(4). doi:10.1371/journal.ppat.1001331

63. Ivashkiv, L. B., & Donlin, L. T. (2014). Regulation of type I interferon responses. *Nature Reviews Immunology,14*(1), 36-49. doi:10.1038/nri3581

64. Kaur, S., & Platanias, L. C. (2013). IFN-β-specific signaling via a unique IFNAR1 interaction. *Nature Immunology,14*(9), 884-885. doi:10.1038/ni.2686

65. Weerd, N. A., Vivian, J. P., Nguyen, T. K., Mangan, N. E., Gould, J. A., Braniff, S., . . . Hertzog, P. J. (2013). Structural basis of a unique interferon-β signaling axis mediated via the receptor IFNAR1. *Nature Immunology,14*(9), 901-907. doi:10.1038/ni.2667

66. Gauzzi, M. C., Velazquez, L., Mckendry, R., Mogensen, K. E., Fellous, M., & Pellegrini, S. (1996). Interferon-α-dependent Activation of Tyk2 Requires Phosphorylation of Positive Regulatory Tyrosines by Another Kinase. *Journal of Biological Chemistry,271*(34), 20494-20500. doi:10.1074/jbc.271.34.20494

67. Marijanovic, Z., Ragimbeau, J., Van Der Heyden, J., Uzé, G., & Pellegrini, S. (2007). Comparable potency of IFNα2 and IFNβ on immediate JAK/STAT activation but differential down-regulation of IFNAR2. *Biochemical Journal,407*(1), 141-151. doi:10.1042/bj20070605

68. Fuchs, S. Y. (2012). Ubiquitination-mediated regulation of interferon responses. *Growth Factors,30*(3), 141-148. doi:10.3109/08977194.2012.669382

69. Kumar, K. G., Krolewski, J. J., & Fuchs, S. Y. (2004). Phosphorylation and Specific Ubiquitin Acceptor Sites Are Required for Ubiquitination and Degradation of the IFNAR1 Subunit of Type I Interferon Receptor. *Journal of Biological Chemistry,279*(45), 46614-46620. doi:10.1074/jbc.m407082200

70. Kumar, K. S., Barriere, H., Carbone, C. J., Liu, J., Swaminathan, G., Xu, P., Fuchs, S. Y. (2007). Site-specific ubiquitination exposes a linear motif to promote interferon-α receptor endocytosis. *The Journal of Cell Biology,179*(5), 935-950. doi:10.1083/jcb.200706034

71. Kumar, K. S. (2003). SCFHOS ubiquitin ligase mediates the ligand-induced down-regulation of the interferon- receptor. *The EMBO Journal,22*(20), 5480-5490. doi:10.1093/emboj/cdg524

72. Kumar, K. G., Varghese, B., Banerjee, A., Baker, D. P., Constantinescu, S. N., Pellegrini, S., & Fuchs, S. Y. (2008). Basal Ubiquitin-independent Internalization of Interferon α Receptor Is Prevented by Tyk2-mediated Masking of a Linear Endocytic Motif. *Journal of Biological Chemistry,283*(27), 18566-18572. doi:10.1074/jbc.m800991200

73. Ragimbeau, J. (2003). The tyrosine kinase Tyk2 controls IFNAR1 cell surface expression. *The EMBO Journal,22*(3), 537-547. doi:10.1093/emboj/cdg038

74. Gakovic, M., Ragimbeau, J., Francois, V., Constantinescu, S. N., & Pellegrini, S. (2008). The Stat3-activating Tyk2 V678F Mutant Does Not Up-regulate Signaling through the Type I Interferon Receptor but Confers Ligand Hypersensitivity to a Homodimeric Receptor. *Journal of Biological Chemistry,283*(27), 18522-18529. doi:10.1074/jbc.m801427200

75. Gilli, F. (2010). Role of Differential Expression of Interferon Receptor Isoforms on the Response of Multiple Sclerosis Patients to Therapy with Interferon Beta. *Journal of Interferon & Cytokine Research,30*(10), 733-741. doi:10.1089/jir.2010.0098

76. Hardy, M. P. (2001). The soluble murine type I interferon receptor Ifnar-2 is present in serum, is independently regulated, and has both agonistic and antagonistic properties. *Blood,97*(2), 473-482. doi:10.1182/blood.v97.2.473

77. Saleh, A. Z., Fang, A. T., Arch, A. E., Neupane, D., Fiky, A. E., & Krolewski, J. J. (2004). Regulated proteolysis of the IFNaR2 subunit of the interferon-alpha receptor. *Oncogene,23*(42), 7076-7086. doi:10.1038/sj.onc.1207955

78. Leang, R. S., Wu, T., Hwang, S., Liang, L. T., Tong, L., Truong, J. T., & Sun, R. (2011). The Anti-interferon Activity of Conserved Viral dUTPase ORF54 is Essential for an Effective MHV-68 Infection. *PLoS Pathogens,7*(10). doi:10.1371/journal.ppat.1002292

79. Madrid, A. S., & Ganem, D. (2012). Kaposis Sarcoma-Associated Herpesvirus ORF54/dUTPase Downregulates a Ligand for the NK Activating Receptor NKp44. *Journal of Virology,86*(16), 8693-8704. doi:10.1128/jvi.00252-12

80. Stefani, F., Zhang, L., Taylor, S., Donovan, J., Rollinson, S., Doyotte, A.,Woodman, P. (2011). UBAP1 Is a Component of an Endosome-Specific ESCRT-I Complex that Is Essential for MVB Sorting. *Current Biology,21*(14), 1245-1250. doi:10.1016/j.cub.2011.06.028

81. Piper, R. C., Dikic, I., & Lukacs, G. L. (2014). Ubiquitin-Dependent Sorting in Endocytosis. *Cold Spring Harbor Perspectives in Biology,6*(1). doi:10.1101/cshperspect.a016808

82. Piper, R. C., & Luzio, J. P. (2007). Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes. *Current Opinion in Cell Biology,19*(4), 459-465. doi:10.1016/j.ceb.2007.07.002

83. Hurley, J. H., & Hanson, P. I. (2010). Membrane budding and scission by the ESCRT machinery: Its all in the neck. *Nature Reviews Molecular Cell Biology,11*(8), 556-566. doi:10.1038/nrm2937

84. Taylor, K. E., & Mossman, K. L. (2013). Recent advances in understanding viral evasion of type I interferon. *Immunology,138*(3), 190-197. doi:10.1111/imm.12038

85. Liu, J., Huangfu, W., Kumar, K. S., Qian, J., Casey, J. P., Hamanaka, R. B., . . . Fuchs, S. Y. (2009). Virus-Induced Unfolded Protein Response Attenuates Antiviral Defenses via Phosphorylation-Dependent Degradation of the Type I Interferon Receptor. *Cell Host & Microbe,5*(1), 72-83. doi:10.1016/j.chom.2008.11.008

86. Qian, J., Zheng, H., Huangfu, W., Liu, J., Carbone, C. J., Leu, N. A., . . . Fuchs, S. Y. (2011). Pathogen Recognition Receptor Signaling Accelerates Phosphorylation-Dependent Degradation of IFNAR1. *PLoS Pathogens,7*(6). doi:10.1371/journal.ppat.1002065

87. Boname, J. M., & Lehner, P. J. (2011). What Has the Study of the K3 and K5 Viral Ubiquitin E3 Ligases Taught Us about Ubiquitin-Mediated Receptor Regulation? *Viruses,3*(2), 118-131. doi:10.3390/v3020118

88. Christ, L., Raiborg, C., Wenzel, E. M., Campsteijn, C., & Stenmark, H. (2017). Cellular Functions and Molecular Mechanisms of the ESCRT Membrane-Scission Machinery. *Trends in Biochemical Sciences,42*(1), 42-56. doi:10.1016/j.tibs.2016.08.016

89. Agromayor, M., Soler, N., Caballe, A., Kueck, T., Freund, S., Allen, M., . . . Williams, R. (2012). The UBAP1 subunit of ESCRT-I interacts with ubiquitin via a novel SOUBA domain. doi:10.2210/pdb4ae4/pdb

90. Rollinson, S., Rizzu, P., Sikkink, S., Baker, M., Halliwell, N., Snowden, J.,…Pickering-Browm, S.M. (2009). Ubiquitin associated protein 1 is a risk factor for frontotemporal lobar degeneration. *Neurobiology of Aging*, 30(4), 656-65. doi:10.1016/j.neurobiolaging.2009.01.009.

91. Xiao, Y., Huang, Y., Xu, P., Zhou, Z., & Li, X. (2006). Pro-apoptotic effect of cecropin AD on nasopharyngeal carcinoma cells. *Chinese Medical Journal,119*(12), 1042-1046. doi:10.1097/00029330-200606020-00014

92. Lang, R., Pauleau, A., Parganas, E., Takahashi, Y., Mages, J., Ihle, J. N., Murray, P. J. (2003). SOCS3 regulates the plasticity of gp130 signaling. *Nature Immunology,4*(6), 546-550. doi:10.1038/ni932

93. Mitani, Y., Takaoka, A., Kim, S. H., Kato, Y., Yokochi, T., Tanaka, N., & Taniguchi, T. (2001). Cross talk of the interferon-alpha/beta signalling complex with gp130 for effective interleukin-6 signalling. *Genes to Cells,6*(7), 631-640. doi:10.1046/j.1365-2443.2001.00448.x

94. Saftig, P., & Klumperman, J. (2009). Lysosome biogenesis and lysosomal membrane proteins: Trafficking meets function. *Nature Reviews Molecular Cell Biology,10*(9), 623-635. doi:10.1038/nrm2745

95. Schulze, H., Kolter, T., & Sandhoff, K. (2009). Principles of lysosomal membrane degradation. *Biochimica Et Biophysica Acta (BBA) - Molecular Cell Research,1793*(4), 674-683. doi:10.1016/j.bbamcr.2008.09.020

96. Fujita, H. (2002). A dominant negative form of the AAA ATPase SKD1/VPS4 impairs membrane trafficking out of endosomal/lysosomal compartments: Class E vps phenotype in mammalian cells. *Journal of Cell Science,116*(2), 401-414. doi:10.1242/jcs.00213

97. Lata, S., Schoehn, G., Jain, A., Pires, R., Piehler, J., Gottlinger, H. G., & Weissenhorn, W. (2008). Helical Structures of ESCRT-III Are Disassembled by VPS4. *Science,321*(5894), 1354-1357. doi:10.1126/science.1161070

98. Stuchell-Brereton, M. D., Skalicky, J. J., Kieffer, C., Karren, M. A., Ghaffarian, S., & Sundquist, W. I. (2007). ESCRT-III recognition by VPS4 ATPases. *Nature,449*(7163), 740-744. doi:10.1038/nature06172

99. Taylor, G. M., Hanson, P. I., & Kielian, M. (2007). Ubiquitin Depletion and Dominant-Negative VPS4 Inhibit Rhabdovirus Budding without Affecting Alphavirus Budding. *Journal of Virology,81*(24), 13631-13639. doi:10.1128/jvi.01688-07

100. Veettil, M. V., Kumar, B., Ansari, M. A., Dutta, D., Iqbal, J., Gjyshi, O., . . . Chandran, B. (2016). ESCRT-0 Component Hrs Promotes Macropinocytosis of Kaposis Sarcoma-Associated Herpesvirus in Human Dermal Microvascular Endothelial Cells. *Journal of Virology,90*(8), 3860-3872. doi:10.1128/jvi.02704-15

101. Myoung, J., & Ganem, D. (2011). Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: Maintenance of tight latency with efficient reactivation upon induction. *Journal of Virological Methods,174*(1-2), 12-21. doi:10.1016/j.jviromet.2011.03.012

102. Tabtieng, T., Degterev, A., & Gaglia, M. M. (2018). Caspase-Dependent Suppression of Type I Interferon Signaling Promotes Kaposis Sarcoma-Associated Herpesvirus Lytic Replication. *Journal of Virology,92*(10). doi:10.1128/jvi.00078-18

103. Zhang, R., Xu, A., Qin, C., Zhang, Q., Chen, S., Lang, Y., . . . Tang, J. (2017). Pseudorabies Virus dUTPase UL50 Induces Lysosomal Degradation of Type I Interferon Receptor 1 and Antagonizes the Alpha Interferon Response. *Journal of Virology,91*(21). doi:10.1128/jvi.01148-17

104. Bisson, S. A., Page, A., & Ganem, D. (2009). A Kaposis Sarcoma-Associated Herpesvirus Protein That Forms Inhibitory Complexes with Type I Interferon Receptor Subunits, Jak and STAT Proteins, and Blocks Interferon-Mediated Signal Transduction. *Journal of Virology,83*(10), 5056-5066. doi:10.1128/jvi.02516-08

105. Chandriani, S., & Ganem, D. (2007). Host Transcript Accumulation during Lytic KSHV Infection Reveals Several Classes of Host Responses. *PLoS ONE,2*(8). doi:10.1371/journal.pone.0000811

106. Glaunsinger, B., Chavez, L., & Ganem, D. (2005). The Exonuclease and Host Shutoff Functions of the SOX Protein of Kaposis Sarcoma-Associated Herpesvirus Are Genetically Separable. *Journal of Virology,79*(12), 7396-7401. doi:10.1128/jvi.79.12.7396-7401.2005

107. Caposio, P., Riera, L., Hahn, G., Landolfo, S., & Gribaudo, G. (2004). Evidence that the Human Cytomegalovirus 46-kDa UL72 protein is not an active dUTPase but a late protein dispensable for replication in fibroblasts. *Virology,325*(2), 264-276. doi:10.1016/j.virol.2004.05.010

108. Hofmann, K., & Falquet, L. (2001). A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. *Trends in Biochemical Sciences,26*(6), 347-350. doi:10.1016/s0968-0004(01)01835-7

109. Li, M., Rong, Y., Chuang, Y., Peng, D., & Emr, S. (2015). Ubiquitin-Dependent Lysosomal Membrane Protein Sorting and Degradation. *Molecular Cell,57*(3), 467-478. doi:10.1016/j.molcel.2014.12.012

110. Sarkar, A., & Zohn, I. (2011). Hectd1 regulates intracellular trafficking of Hsp90 to control its secretion and cell motility of the cranial mesenchyme. *Developmental Biology,356*(1), 120. doi:10.1016/j.ydbio.2011.05.064

111. Li, X., Zhou, Q., Sunkara, M., Kutys, M. L., Wu, Z., Rychahou, P., . . . Huang, C. (2013). Ubiquitylation of phosphatidylinositol 4-phosphate 5-kinase type I by HECTD1 regulates focal adhesion dynamics and cell migration. *Journal of Cell Science,126*(12), 2617-2628. doi:10.1242/jcs.117044

112. Tran, H., Bustos, D., Yeh, R., Rubinfeld, B., Lam, C., Shriver, S., . . . Polakis, P. (2012). HectD1 E3 Ligase Modifies Adenomatous Polyposis Coli (APC) with Polyubiquitin to Promote the APC-Axin Interaction. *Journal of Biological Chemistry,288*(6), 3753-3767. doi:10.1074/jbc.m112.415240

113. Zohn, I. E., Anderson, K. V., & Niswander, L. (2007). The Hectd1 ubiquitin ligase is required for development of the head mesenchyme and neural tube closure. *Developmental Biology,306*(1), 208-221. doi:10.1016/j.ydbio.2007.03.018

114. Kerppola, T. K. (2008). Bimolecular Fluorescence Complementation (BiFC) Analysis as a Probe of Protein Interactions in Living Cells. *Annual Review of Biophysics,37*(1), 465-487. doi:10.1146/annurev.biophys.37.032807.125842